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(54) Title: SUBUNIT VACCINE FOR *STREPTOCOCCUS EQUI*

(57) Abstract

The invention relates to a fibrinogen-binding protein of *Streptococcus equi*, to a DNA fragment encoding this protein and to the use of the purified fibrinogen-binding protein or suitable truncate in the preparation of a vaccine against *S. equi* infection, also known as Strangles, in horses.

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Subunit Vaccine for *Streptococcus Equi*

The invention relates to a fibrinogen-binding protein of *Streptococcus equi* and to the use of the purified protein in the preparation of a vaccine against *S. equi* infection in horses.

Strangles is one of the most important infectious diseases affecting horses. It is of major economic importance to the racing and thoroughbred industry. Strangles is caused by the bacterium *Streptococcus equi* also known as *Streptococcus equi* subsp. *equi* (a group C streptococcus), affects the upper respiratory tract and is highly contagious. Horse to horse spread often leads to large outbreaks with many animals infected. Even horses with mild forms of the disease must be isolated and removed from training, stud or other heavy work for up to three months. Existing vaccines have poor efficacy (Yelle, 1987; Timoney, 1988). The present invention involves the development of a novel and efficacious vaccine against the disease and is based on a novel, high molecular-weight (M_r) protective antigen which has fibrinogen-binding properties i.e. an M-like protein.

The main protective antigen of *S. equi* is thought to be an M protein. The best characterized M proteins are those of the group A streptococcus, *Streptococcus pyogenes*. M proteins are members of a broad family of proteins from Gram-positive bacteria. These proteins are associated with the cell wall and are thus generally called cell-wall-associated proteins. They are surface exposed and usually interact with various factors present in the extracellular matrix of mammalian tissues and in body fluids. These factors include collagen, complement components, antibodies, fibrinogen (Fg), fibronectin, kininogen, laminin, 2-macro-globulin, plasmin, prothrombin and salivary glycoproteins. Cell-wall-associated proteins, although quite distinctive, share some common features. Firstly, these proteins are usually α-helical coiled-coil dimers, the N-terminal halves of which project from the cell as fibrillar-type structures. Secondly, the N-terminal signal sequences usually show significant homologies. In addition, the C-terminal regions which are responsible for anchoring these proteins to the bacterial cell wall and membrane also show some homology. For example, there is usually a pro/gly rich region followed by a highly conserved Lys-Pro-X-Thr-Gly-X (LPXTGX) motif, a stretch of about 20 hydrophobic residues and a short tail

of predominantly charged residues. Finally, many of these proteins possess tandemly repeated sequences. Where a protein shows a number of distinct repeats, these are normally distinguished by letters e.g. A repeats, B repeats etc. (Fischetti, 1991; Goward et al., 1993).

Historically, M protein was a name given to a group of proteins of *S. pyogenes*. These proteins conferred on the bacterium the ability to resist phagocytic killing and they were also thought to be involved in adhesion to host surfaces. These two properties are thought to be due in part to two characteristic binding functions of M proteins i.e. the ability to bind fibrinogen and complement factor H. In addition to these binding functions, M proteins also undergo phase and antigenic variation, and up to 80 different serotypes have been discerned (Fischetti, 1991).

Protective M proteins have also been detected in *S. equi*. All the available evidence suggests that immunity to this protein(s) may be important in protection (Galán and Timoney, 1985). Several different methods have been used to extract the M-protein from *S. equi* cells (Boschwitz et al., 1991). These methods include hot acid treatment of whole cells, hot alkaline treatment of whole cells, treatment of whole cells with 4% sodium dodecyl sulphate, ammonium sulphate precipitation of culture supernatants and mutanolysin extraction of bacterial protoplasts. These preparations have been analysed in Western immunoblotting experiments using convalescent horse serum, nasopharyngeal mucus, antiserum to mutanolysin-extracted protein and antiserum to a 41-kDa fragment from acid extracts (Galán & Timoney, 1985; Timoney & Trachman, 1985; Galán & Timoney, 1988; Boschwitz, 1991; Timoney & Mukhtar, 1993). All extracts contain multiple reactive protein bands in the molecular mass range 25-140 kDa. In acid extracts, the most immunologically reactive bands have molecular masses of 46 kDa, 41 kDa and 29 kDa. In mutanolysin extracts, there are two reacting bands in the 58-kDa region and a higher molecular mass band of 120 kDa. The 58-, 46-, 41- and 29-kDa proteins possess the same N-terminal amino acid sequence (data not shown in Timoney & Mukhtar, 1993). Timoney's group suggest that the 58-kDa protein is the native M-protein, that the lower molecular weight reacting bands are peptide fragments i.e. degradation products of M-protein, and that the high molecular mass protein of 120-140 kDa is M-protein complexed to the cell wall (Galán & Timoney, 1985; Timoney et al., 1991; Timoney & Mukhtar, 1993).

Like M-protein of *S. pyogenes*, the M-protein of *S. equi* has been implicated in inhibition of phagocytosis. Recent evidence suggests that resistance to phagocytosis may be related to the ability of M-protein to interfere with the deposition of equine complement on the cell surface (Boschwitz & Timoney, 1994a). These authors have also provided some additional evidence that binding of fibrinogen to *S. equi* cells has some anti-phagocytic activity. From a study of non-isogenic strains, they further suggest that the M-protein may bind fibrinogen since a strain of *S. equi* expressing low levels of M-protein bound 64% less fibrinogen than another strain expressing normal M-protein levels (Boschwitz & Timoney, 1994b). However, binding of fibrinogen to a defined molecular species has not been shown.

A mouse model has also been used to test the protective potential of streptococcal extracts and of immunoglobulin preparations. Acid-extracted M-protein (containing the mixture of immunologically-reactive species described above) and monoclonal antibodies generated to this preparation have been shown to afford 60-80% protection in a mouse model (Timoney & Trachman, 1985; Jean-Francois et al., 1991). Efforts were also undertaken to clone the gene encoding M-protein (Galán & Timoney, 1987, Timoney et al., 1991). Galán & Timoney (1987) isolated clones from a recombinant phage bank which reacted with opsonic and anti-M protein antiserum and expressed proteins of M_r 58,000, 53,000 and 50,000. In other publications (Galán & Timoney, 1988; Timoney et al., 1991), two plasmid subclones from the phage library were described and about 550 bp of partial sequence was published.

At this stage it is perhaps worth reemphasizing that the principle group working in the field of M-proteins of *S. equi* (i.e. that of Timoney) considers the 58-kDa protein antigen to be the native M-protein.

In contrast to the M-protein of *S. pyogenes* a variety of evidence suggests that there is only one serotype of M-protein from *S. equi*. This includes evidence from precipitin and passive protection experiments, sensitivity to bactericidal serum, immunoblot and M_r analysis, DNA restriction analysis and Southern blot analysis (see Galán & Timoney, 1988).

Existing vaccines against strangles are based on crude bacterins (heat-killed *S. equi)* or acid extracts enriched in the M-protein. U.S.

Patent No. 3,852,420 is concerned with extracting immunity-provoking antigen by hot acid treatment of cells. U.S. Patent No. 4,582,798 of Brown et al., is concerned with an alternative technique for extracting the antigen of interest by treatment of the cells with mutanolysin and an anionic detergent. U.S. Patent No. 5,183,659 of Timoney relates to a vaccine comprising an avirulent strain of *S. equi*. Existing vaccines include Equibac (Fort Dodge Labs., Iowa), Strangles vaccine (Commonwealth Serum Labs., Melbourne), Strepguard (Haver Labs., Kansas), Strepvax II (Cooper Animal Health, Kansas) and Stranglevac (Bayer Animal Health, Kansas/Miles Inc., Slough, U.K.). These vaccines are administered intramuscularily with adjuvant. They cause some side effects and more importantly afford little protection (Yelle, 1986; Timoney, 1988). Galán & Timoney (1985) showed that there was no correlation between protection and the levels of serum bactericidal antibodies directed against *S. equi* whole cells. The authors hypothesize that immunity to infection is mediated by locally produced mucosal nasopharyngeal antibodies and that the lack of efficacy of existing vaccines is due to the failure to stimulate this kind of response. Indeed, Brown & Bryant, 1990 (U.S. Patent No. 4,944, 942) provide convincing evidence that intranasal immunization can protect horses from experimental challenge with *S. equi*. The antigenic material to be used in this invention can be inactivated whole organisms or appropriate extracts prepared from these organisms or recombinant DNA or synthetic peptides. It is stated that the preferred technique for preparing antigenic material is by enzyme extraction using enzymes such as pepsin, lysozyme or mutanolysin and to follow the enzyme extraction with treatment with an anionic detergent such as SDS.

The object of the present invention is to provide a cheap, effective vaccine against *S. equi* infection or strangles of horses.

A further object is to provide a defined subunit vaccine against strangles. It is also an object to isolate a protective cell-wall-associated protein from *S. equi*, and to purify this protein to homogeneity. An additional objective is to isolate the gene encoding the said protein.

According to the present invention there is provided a fibrinogen-binding protein (FgBP) isolated from *S. equi* subspecies *equi* having the following characteristics:-

(1) it migrates on Laemmli SDS-PAGE gels with an apparent molecular weight of approximately 220,000,

(2) it binds horse fibrinogen, and

(3) it protects against *S. equi* infection (Strangles) in horses.

The fibrinogen-binding protein is preferably associated with the cell wall of *S. equi*.

The fibrinogen-binding protein may be a multimeric alpha-helical coiled-coil structure, the individual monomers having a molecular weight of approximately 55,000.

Preferably the fibrinogen-binding protein comprises an amino acid sequence selected from (1) NSEVSRTATPRL and (2) LQKAKDERQALTESFNKTLS.

The invention also provides an *S. equi* fibrinogen-binding protein which comprises the nucleotide sequence as shown in Figures 15 or 18.

The invention further provides a DNA fragment selected from fragments as deposited at The National Collections of Industrial and Marine Bacteria, St. Machar Drive, Aberdeen, Scotland under the Accession Numbers NCIMB 40807 on 25th June 1996, and NCIMB 40883 on 19th June 1997 encoding the *S. equi* fibrinogen-binding protein or a fragment substantially similar thereto also encoding *S. equi* fibrinogen-binding protein and/or Strangles protective activity.

The invention further relates to a host cell comprising a DNA fragment as described above and to a method of producing a fibrinogen-binding protein comprising culturing a host cell containing the said DNA fragment and isolating the protein from the culture.

In a further aspect the invention provides a method of producing an *S. equi* fibrinogen-binding protein comprising the steps:-

(1) genetically engineering a host cell containing the DNA fragment defined above to overexpress and/or secrete the fibrinogen-binding protein (or suitable truncate) into the culture supernatant,

(2) isolating the fibrinogen-binding protein in a cell-free supernatant fraction, and

(3) purifying the fibrinogen-binding protein.

By suitable truncate is meant a fibrinogen-binding protein derivative which is lacking the C-terminal segment responsible for anchoring the protein to the cell envelope. Such a truncate would not be capable of binding to the cell wall since it would be lacking its cell wall/membrane anchor domains, and would thus be secreted into the supernatant, simplifying the purification procedure.

The fibrinogen-binding protein or truncate may be isolated in a cell-free supernatant fraction by either (a) lysis of the host cell followed by centrifugation or by any of a number of filtration methods known in the art, or by (b) isolation of the culture supernatant by centrifugation or by any of a number of filtration methods known in the art.

The fibrinogen-binding protein or truncate may be purified by fibrinogen- affinity chromatography or other chromatographic procedures known in the art.

The invention further provides a vaccine comprising a fibrinogen-binding protein as defined above or whenever produced by a method as described above.

The invention also provides an *S. equi* fibrinogen-binding protein as defined above for use in the preparation of a vaccine against Strangles infection in horses.

By "substantially similar" herein is meant DNA fragments encoding fibrinogen-binding activity which have sufficient sequence identity or homology to the deposited DNA fragments, by virtue of the degeneracy of the genetic code or by virtue of mutation, to hybridise therewith and to bind fibrinogen and to protect against *S. equi* infection or Strangles in horses.

The invention will be described further with reference to the drawings in which there is shown:

Figure 1. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and fibrinogen-affinity blotting analysis of proteins released from purified cell walls of *S. equi* following treatment with mutanolysin. Samples were boiled at 100°C in Laemmli sample buffer and analysed by SDS-PAGE using a 12.5% (wt/vol) polyacrylamide separation gel (Laemmli, 1970). Lane 1A and 1B, cell envelopes following extraction with 2% (wt/vol) SDS; lane 2A and 2B, supernatant fraction from purified cell walls following incubation with mutanolysin; lane 3A and 3B, supernatant fraction from purified cell walls incubated without mutanolysin. The SDS-gel in Panel A was stained with Coomassie-brilliant blue. Panel B shows an identical gel electrotransferred onto nitrocellulose and affinity probed with horseradish peroxidase conjugated-horse fibrinogen. Positions and molecular masses of mutanolysin-extracted proteins and of mutanolysin are indicated at the right of panel A. The position of the fibrinogen-reacting 220-kDa protein is indicated by arrowhead at the right of Panel B. Molecular masses were determined from the relative mobilities of the following standard molecular mass marker proteins: rabbit myosin (205 kDa), ß-galactosidase (116 kDa), phosphorylase b (94 kDa), bovine serum albumin (66.2 kDa), catalase (61 kDa), glutamate dehydrogenase (55.4 kDa), fumarase (48.5 kDa), alcohol dehydrogenase (41 kDa), Omp F protein of *E. coli* (36.5 kDa), carbonic anhydrase (30 kDa), chymotrypsinogen (25.1 kDa), trypsin inhibitor (20.1 kDa), lysozyme (14.3 kDa).

Figure 2. Analysis by SDS-PAGE of the purification of the FgBP from *S. equi*. Samples solubilized at 100°C in Laemmli sample buffer and analysed by SDS-PAGE using a 12.5% (wt/vol) polyacrylamide separating gel (Laemmli, 1970). Lane 1, cell envelope fraction; lane 2, cell envelope fraction following incubation with mutanolysin; lane 3, pellet obtained following incubation of cell envelopes with mutanolysin; lane 4, supernatant fraction obtained following incubation of cell envelopes with mutanolysin; lane 5, proteins eluted unbound from the fibrinogen affinity column; lane 6, FgBP purified by fibrinogen-affinity chromatography. The position of the FgBP is indicated. Indicated to the left of the SDS-gel are the positions (in kilodaltons) to which standard molecular mass marker proteins migrated.

Figure 3. Protective effect of FgBP against lethal *S. equi* infection in mice. (a) Twenty one, and seven days prior to challenge, a group of 11 mice were immunized (subcutaneously) with partially purified FgBP

emulsified in MPL+S-TDCM Ribi adjuvant. A control group of mice were immunized (subcutaneously) with MPL+S-TDCM Ribi adjuvant emulsified in phosphate-buffered saline (PBS). All mice were subsequently challenged with 3 x 10^5 colony forming units (CFU) of *S. equi* cells. (b) Forty two and fourteen days prior to challenge, a group of 10 mice were immunized (subcutaneously) with affinity-purified FgBp emulsified in MPL+S-TDCM Ribi adjuvant. Control mice were immunized with PBS only or with MPL+S-TDCM Ribi adjuvant emulsified with PBS. All mice were subsequently challenged with 1.5 x 10^5 CFU of *S. equi* cells. Mice were sacrificed if considered terminally ill with body temperatures below 32°C. ⊡ controls; ◆ vaccinates.

Figure 4. Serum IgG response, as monitored by ELISA, of mice vaccinated with FgBP and of control unvaccinated mice. The log of serum dilutions versus the mean absorbance readings of the vaccinated (◆) group of mice and of the control (⊡) group of mice are shown.

Figure 5. Numerical clinical scoring system used in the equine trials.

Figure 6. Total daily clinical scores during equine trial. Days of vaccination (V) and challenge (C) are indicated. ⊡ denotes horse H1; ○ denotes horse H2; ■ denotes horse H3.

Figure 7. Daily temperature score during equine trial. Days of vaccination (V) and challenge (C) are indicated. ⊡ denotes horse H1; ○ denotes horse H2; ■ denotes horse H3.

Figure 8. Daily fibrinogen levels and white blood cell counts for horse H1 during equine trial. Days of vaccination (V) and challenge (C) are indicated. ⊡ denotes fibrinogen level; ◆ denotes white blood cell counts.

Figure 9. Daily fibrinogen levels and white blood cell counts for horse H2 during equine trial. Days of vaccination (V) and challenge (C) are indicated. ⊡ denotes fibrinogen level; ◆ denotes white blood cell counts.

Figure 10. Daily fibrinogen levels and white blood cell counts for horse H3 during equine trial. Days of vaccination (V) and challenge (C) are

indicated. ⊡ denotes fibrinogen level; ◆ denotes white blood cell counts.

Figure 11. Serum IgG titres against FgBP for horses H1, H2 and H3 taken on days post primary vaccination. Days of vaccination (V) and challenge (C) are indicated. ⊡ denotes horse H1; ○ denotes horse H2; ■ denotes horse H3.

Figure 12. Nasal mucosa (secretory) IgG titres against FgBP for horses H1, H2 and H3 taken on days post primary vaccination. Days of vaccination (V) and challenge (C) are indicated. ⊡ denotes horse H1; ○ denotes horse H2; ■ denotes horse H3.

Figure 13. Oligonucleotide probe used in Southern blots to screen for the gene encoding the FgBP. The corresponding amino acid sequence is shown above in single letter code.

Figure 14. Genotypes of bacterial strains.

Figure 15. Nucleotide and deduced amino acid sequence of the 5' region of the gene encoding the FgBP of *S. equi*. The probable start of the signal sequence is arrowed. The sequences corresponding to that determined by direct amino acid sequence analysis of the V8 protease fragments are underlined.

Figure 16. Partial restriction map of pFBP200. The open box represents the multiple cloning site in *lacZ* of pGEM7. The thin line represents λGEM11 DNA. The thicker line represents *S. equi* DNA. The region which has been sequenced, in pFBP100, has a bar beneath it. The region of DNA encoding the truncated *fbp* gene is indicated by an arrow.

Figure 17. Partial restriction map for the insert in pFBP700. The thick line represents the coding region for the FgBP and an arrow indicates the direction of transcription. The directions of the universal forward (F) and reverse (R) primers present in pBK-CMV are also shown.

Figure 18. Nucleotide sequence and deduced amino acid sequence of the gene *(fbp)* encoding the FgBP. A putative ribosome binding site (RBS) and possible -10 and -35 promoter signals are underlined. The putative signal

peptidase cleavage site is marked with an arrow, the stop codon is marked with an asterick and a possible transcriptional termination hairpin loop is underlined with arrowheads. Underlined amino acids correspond to the sequences determined by direct amino acid sequence analysis of the V8 protease fragments. The repeat regions (A1, A2, A3, B1 and B2), the consensus LPSTGE motif (in bold), membrane anchoring domain (M) and the charged tail (C) are also indicated.

Figure 19. **Purification of hexahistidyl-tagged FgBP truncate** analysed by SDS-PAGE and fibrinogen-affinity blotting. Samples were solubilized in Laemmli sample buffer and analysed by SDS-PAGE using a 12.5% (wt/vol) polyacrylamide separating gel (Laemmli, 1970). Lane 1, soluble fraction of *E. coli* harbouring pQE30; lane 2, soluble fraction of *E. coli* harbouring pQE30-*fbp;* lane 3 and 5, proteins eluted from the Ni^{2+}-IDA column with 100 mM imidazole; lanes 4 and 6, proteins eluted from the Ni^{2+}-IDA column with 200 mM imidazole. Lanes 1-4 were stained for protein with Coomassie brilliant blue. Lanes 5-6 were electrotransferred onto nitrocellulose and affinity probed with horseradish peroxidase conjugated-horse fibrinogen. The position of the 220-kDa and 90-kDa FgBP truncates are indicated to the right. Indicated to the left of the SDS-gel are the positions (in kilodaltons) to which molecular mass marker proteins migrated.

Results

Isolation of cell wall-associated proteins

In order to identify candidate cell-wall-associated proteins from *S. equi*, it was first necessary to separate these proteins from other membrane proteins. This involved purification of cell-wall material, degradation of cell-wall polymers and isolation of proteins specifically released from the degraded cell wall. These steps are outlined below. *S. equi* cells were grown to late logarithmic phase in Todd-Hewitt broth supplemented with 0.2% (wt/vol) yeast extract. Usually about 6 litres of cells were grown at a time, the yield of cells being about 2 g wet weight per litre of broth. Bacterial cells were harvested by centrifugation (16,000 x g for 15 min), washed once in 10mM Tris-HCl buffer pH 7.2 (Tris buffer) and finally resuspended in 120 ml Tris buffer containing DNase (50µg/ml), RNase (50 µg/ml) and protease inhibitors (phenylmethylsulfonyl-

floride [2 mM], and benzamidine hydrochloride [2 mM]. The bacteria were then lysed by two passages through a French pressure cell (32,000 lb/in^2). Unlysed cells were removed from the cell lysate by centrifugation (3,000 x g for 10 min). Cell wall-membranes (cell envelopes) were pelleted from the cleared lysate by centrifugation (45,000 x g for 1 h) and washed three times in Tris buffer. The cell envelopes were then resuspended in 36 ml of Tris buffer containing 2% (wt/vol) sodium dodecyl sulphate (SDS) and incubated at 20°C for 1 h. Purified cell-wall material (plus associated polymers and proteins) was obtained as an SDS-insoluble (pellet) fraction following centrifugation (45,000 x g for 1 h). The SDS-extraction was repeated once more as outlined above. The SDS-insoluble pellet was washed 5 times in Tris buffer, once in 10 mM sodium phosphate buffer, pH 7.2, and finally resuspended in 9 ml of 10 mM sodium phosphate buffer, pH 7.2. The purified cell wall was then digested by incubation for 18 h at 37°C with mutanolysin (Sigma; 18,000 U), in the presence of protease inhibitors (Nα-*p*-tosyl-L-lysinechloromethylketone [2mM], phenylmethylsulfonyl- floride [2 mM], and benzamidine hydrochloride [2 mM]). The extract was centrifuged (45,000 x g for 1 h) and material released from the digested cell wall was obtained in the supernatant fraction. These various fractions were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The results showed that several proteins were specifically released from the digested peptidoglycan. These proteins included one major protein of apparent M_r220,000. Other proteins of apparent M_r94,000, 74,000 and 56,000 and minor species of M_r44,000, 38,000 and 29,000 were also detected (Figure 1, lane 2A). It can be concluded that these proteins are likely to be associated with the cell wall.

Fibrinogen-binding ability of the 220-kDa protein

An affinity blotting assay of the above preparation of cell-wall-associated proteins was performed using horse fibrinogen which had been labelled with horseradish peroxidase.

Removal of contaminating fibronectin and IgG from horse fibrinogen

Prior to conducting the affinity blotting assay the major contaminating serum proteins (fibronectin and immunoglobulin G) were first removed from the preparation of horse fibrinogen by gelatin affinity chromatography (Mosher et al., 1980) and by protein G affinity

chromatography (Pierce Chemical Co.). In these procedures, contaminating fibronectin was first removed using a gelatin-affinity column (Mosher et al., 1980) prepared by covalently linking gelatin to CnBr-activated Sepharose 4B™ using the method recommended by the manufacturer (Pharmacia LKB Biotechnology). Horse fibrinogen (10 mg/ml in 50 mM sodium phosphate, 1 mM ethylenediaminetetraacetate (EDTA) and 0.5M NaCl, pH 7.5) was then applied to the gelatin column. Fibronectin remained bound to the column, whereas fibrinogen was eluted as unbound material. The eluate was then dialysed against 100 mM sodium acetate (pH 5.0), and a precipitate of lipoproteins removed by centrifugation (16,000 x g for 15 min). Contaminating horse IgG was then removed from the supernatant fraction using a protein G/agarose affinity column and following the procedure recommended by the manufacturer (Pierce Chemical Co.). Horse IgG remained bound to the column whereas fibrinogen was eluted as unbound material. Purified fibrinogen (free of fibronectin and IgG) was then dialysed against distilled water and lyophilised.

Conjugation of fibrinogen with horseradish peroxidase

Purified fibrinogen was labelled with horseradish peroxidase based on a method described by Winston et al. (1995). This entailed firstly treatment of horseradish peroxidase (5 mg/ml) with 40 mM $NaIO_4$ in the dark for 30 min at 20°C. Ethylene glycol was then added to a final concentration of 0.64 M and the solution incubated for a further 1 h before dialysis versus 1 M sodium acetate buffer (pH 4.4). Lyophilised fibrinogen (25 mg) was the dissolved in the above solution of horseradish peroxidase. The pH of the solution was adjusted to 9.5 using 100 mM sodium carbonate buffer (pH 9.5) and the mixture was incubated in the dark for 1h at 20°C. 0.1 ml of aqueous $NaBH_4$ (4 mg/ml) was added and the solution incubated for a further 1 h in order to reduce reactive groups. Peroxidase-labelled fibrinogen was then dialysed extensively against phosphate buffered saline (pH 7.2; PBS) and finally stored at -20°C.

Horse fibrinogen affinity-blotting assay

Affinity blotting assays were performed in a similar manner to that described for Western immunoblotting (Caffrey et al., 1988). 2% (wt/vol) dried skimmed milk was used as a blocking reagent. Bound peroxidase-conjugated horse fibrinogen was visualized with 4-chloro-1-naphthol. The

results of the affinity blot showed that the protein of apparent M_r of 220,000 bound horse fibrinogen (Figure 1B).

Purification of the fibrinogen-binding protein

The fibrinogen-binding protein (FgBP) of apparent M_r 220,000 was purified to homogeneity using fibrinogen-affinity chromatography. The purification steps are outlined below and an SDS polyacrylamide gel of the various purification steps are shown in Figure 2. Bacterial cell envelopes (i.e. cell wall-membranes which had not been extracted in SDS) were isolated by a procedure outlined in the Section entitled "Isolation of cell wall-associated proteins". Isolated cell envelopes were then washed three times in Tris buffer, once in 10 mM sodium phosphate buffer (pH 6.8) and finally resuspended in 12 ml of sodium phosphate buffer containing mutanolysin (9600 U) and protease inhibitors (Nα-*p*-tosyl-L-lysinechloromethylketone [2mM], phenyl-methylsulfonylfloride [2 mM], and benzamidine hydrochloride [2 mM]). The suspension was extracted at 37°C for 18 h and then centrifuged (45,000 x g for 1 h). Material released from the digested cell envelope and thus obtained in the supernatant fraction was analysed by SDS-PAGE (see Figure 2). The profile of proteins, which were released into the supernatant fraction, was identical to that released following mutanolysin extraction of purified cell walls (see Section entitled "Isolation of cell wall-associated proteins" and Figure 1). The supernatant fraction of the mutanolysin extract was then incubated, with shaking, for 2 h with approximately 7 ml of fibrinogen-sepharose 4B prepared by covalently linking horse fibrinogen to CnBr-activated Sepharose 4B™ by a method recommended by the manufacturer (Pharmacia LKB Biotechnology). The fibrinogen-sepharose 4B slurry was poured into a 1 x 9 cm chromatography column, and eluted with 10 mM sodium phosphate buffer (pH 6.8) until the OD_{280nm} approached zero. 0.2 M glycine (pH 2.5) was used to elute bound FgBP. Fractions were collected into 1M Tris-HCl (pH 8.0) and analysed by SDS-PAGE. Those containing FgBP were pooled, dialysed against PBS and lyophilised.

Assessment of the protective potential of FgBP in a mouse model

To assess the vaccinogenic potential of the FgBP, two separate protection experiments were performed in a mouse model.

S. equi cells used for challenge

S. equi cells used for challenge of the mice were grown as described in the Section entitled "Isolation of cell wall-associated proteins". Harvested cells were washed twice in sterile PBS (1/5 culture volume), resuspended in PBS (1/100 culture volume) and suspensions were stored in aliquots at -70°C. *S. equi* cells stored in this manner maintained viability for several months. On the day of challenge, an aliquot of frozen cells was thawed and diluted appropriately in PBS.

Mouse challenge experiments

In the first of these experiments, the protective immunogen was a preparation of FgBP which had been isolated following mutanolysin extraction of purified cell walls and further purified (to approximately 80% homogeneity) by gel filtration chromatography. Eleven mice were immunized subcutaneously on days 0 and 14 with 100 µg protein emulsified in MPL+S-TDCM Ribi adjuvant (active ingredients monophosphoryl lipid A and trehalose dimycolate; RIBI Immunochem Research, Inc.). Ten control mice were immunized with adjuvant emulsified in PBS only. All mice were challenged on day 21 by intraperitoneal injection of 3×10^5 colony forming units (CFU) of virulent *S. equi* and were monitored for 32 days post challenge. In this study, mortality of vaccinated mice was reduced by 50% and moreover, the mean time to death was extended from 3.5 days for unvaccinated mice to 10.5 days for vaccinated mice (see Figure 3a).

In the second protection study, 10 mice were immunized subcutaneously on days 0 and 28 with 50 µg of affinity-purified FgBP (see Section entitled "Purification of the fibrinogen-binding protein") emulsified in MPL+S-TDCM Ribi adjuvant. 5 control mice were immunized with PBS only and 5 mice were immunized with MPL+S-TDCM Ribi adjuvant emulsified in PBS. All mice were challenged on day 42 with 1.5×10^5 CFU of *S. equi*. In this experiment, all vaccinates were protected following challenge of a lethal dose of *S. equi* (see Figure 3b). Furthermore, at no stage did any vaccinated mouse show any clinical signs of illness. In contrast, mortality in the control group of mice was 80%.

Serum IgG response to FgBP

Enzyme-linked immunosorbent assays (ELISA) were performed on sera taken from vaccinated and control mice prior to challenge in order to determine the serum IgG response to FgBP. Assays were carried out using standard procedures (Newell et al., 1988). Briefly, microtitre plates were filled with 50 µl volumes of purified FgBP solution (50.4 ng/µl of 100 mM sodium carbonate buffer, pH 9.6) and incubated overnight at 20°C. Coated-wells were blocked by incubation for 45 min with 50 mM Tris acetate buffer pH, 7.4 containing 1% (wt/vol) bovine serum albumin, 0.05% (vol/vol) Tween 20 and 0.9% (wt/vol) NaCl and washed three times with 0.05% (vol/vol) Tween 20 in 0.9% (wt/vol) NaCl (wash buffer). The wells were incubated for 2 h with serial two-fold dilutions of sera prediluted 1:250 in blocking solution and then washed three times in wash buffer. The wells were incubated for a further 1 h with 1:2000 dilution of peroxidase-labelled affinity-purified anti-mouse IgG (H+L) and washed again in wash buffer. 3,3'5,5'-tetramethylbenzidine was used as substrate and absorbance readings were measured at 450nm. All assays were performed in duplicate. Background readings were determined from the use of uncoated wells which were processed in an identical fashion to those coated with FgBP.

Results (Figure 4) show that the vaccinated mice had a high level of serum antibody to the 220-kDa FgBP whereas the control mice did not. This was confirmed by Western immunoblots conducted using the above sera (data not shown).

These data show that purified FgBP offers significant protection to mice against lethal challenge by *S. equi*.

<u>Vaccinogenic potential of FgBP against S. equi infection in horses</u>

In view of the encouraging results obtained in the mouse challenge experiments, the vaccinogenic potential of purified FgBP was tested in the host species for *S. equi*, the horse.

Vaccine

FgBP was purified using fibrinogen-affinity chromatography as described in the Section entitled "Purification of the fibrinogen-binding

protein".

Challenge

The challenge organism, *S. equi*, was grown, washed and stored as described in the Section entitled "Assessment of the protective potential of FgBP in a mouse model".

Experimental animals

Two thoroughbred male horses (2-3 years) and one male pony (12 years) were used in the trial. None had a previous history of strangles.

Vaccination and challenge

Two horses, termed H1 (a thoroughbred) and H2 (the pony), were vaccinated with FgBP intranasally three times at approximately three week intervals i.e. on days 0, 21 and 42. Each vaccination consisted of 500 µg of FgBP in 4 ml PBS. One control horse, termed H3 (a thoroughbred) was immunized by the same protocol using PBS alone. Nineteen days after the final booster (day 61), all three horses were challenged intranasally with 1×10^7 CFU of *S. equi* in 4 ml of PBS.

Intranasal vaccination and challenge were administered using a dog urinary catheter (50 cm length x 2.0 mm diameter) attached at the proximal end to a 20-ml syringe. The distal end of the dog catheter was heat-sealed and about 15-20 small holes (<1 mm in diameter) were made around its circumference. The entire length of the catheter was inserted into the horse's nose and the fluid sprayed into the tonsillar region. A 2-ml volume was delivered in this manner up each nostril.

Evaluation of response to vaccination and challenge

The clinical response of the horses to vaccination and challenge was determined on a daily or every-other-day basis, using the clinical scoring system outlined in Figure 5. Blood samples and two nasal swabs were taken at weekly intervals. Blood samples were subject to routine haematological and biochemical tests as well as ELISA tests to monitor serum IgG to FgBP. In addition, nasal swabs were analyzed for microbial flora and by ELISA for

secretory IgG and IgA to FgBP.

Haematology, blood biochemistry and microbiology

Haematological, biochemical and microbiological tests were performed by the Irish Equine Centre, Johnstown, Co. Kildare, Ireland. Blood haematology tests included those for red blood cell, packed cell volume, haemoglobin, mean cell volume, mean corpuscular haemoglobin content, platelets, white blood cell, fibrinogen, neutrophils, lymphocytes, monocytes and eosinophils. Blood biochemical tests included those for total protein, albumin, aspartate aminotransferase, creatinine kinase, gamma glutamyltransferase, total bilirubin, sodium and potassium levels. Microbiological analysis included routine tests for the presence of *Staphlycoccus aureus*, alpha-haemolytic streptococci, ß-haemolytic streptococci, *Neisseria* sp., *Bacteroides* sp., *Clostridium* sp., non-haemolytic *Escherichia coli* and *Streptococcus equi*.

Processing of nasal swabs for ELISA assays

Nasal swabs were individually immersed in 1 ml of saline for 18 h at 4°C. Samples were then vortexed vigorously for 2 min and the saline removed. The swab was then vortexed in a further 0.5 ml of saline. The final volume of the combined nasal swab washings was then adjusted to 1.5 ml, the washings were centrifuged at 13,000 x g for 10 minutes and the supernatant fluids were stored at -20°C until use.

ELISA of serum and secretory immunoglobulin to FgBP

ELISA assays were carried out by standard procedures essentially as described in the Section entitled "Assessment of the protective potential of the FgBP in the mouse model". After coating with FgBP and washing, wells were incubated for 2 h with serial two-fold dilutions of either sera (prediluted 1:500 in blocking buffer) or nasal swab washings. After rinsing the wells in wash buffer, the wells were incubated for 1 h with either a 1:2000 dilution of peroxidase-labelled affinity-purified anti-horse IgG (H+L) or a 1:2000 dilution of peroxidase-labelled affinity-purified anti-horse IgA (alpha-chain specific). 3,3'5,5'-tetramethylbenzidine was used as substrate and absorbance readings were measured at 450nm. All assays were performed in triplicate. The

ELISA endpoint titre was regarded as the reciprocal of the serum or nasal swab washing which gave an OD_{450nm} of 0.1 and was calculated graphically. Background readings were determined from the use of uncoated wells which were processed in an identical fashion to those coated with FgBP. Titres obtained from the use of nasal swab washings were normalised with respect to total protein.

Results

The total clinical scores of the three horses are shown in Figure 6. Of the 16 individual clinical symptoms incorporated in the total clinical scores (Figure 5), temperature is considered to be the best indicator of forthcoming illness and is highlighted in Figure 7. Two other relevant (blood) parameters i.e. fibrinogen levels and white blood cell counts, which are indicative of infection and inflammation are shown in Figures 8, 9 and 10. The immunoglobulin titres of the sera and nasal swab washings are shown in Figures 11 and 12, respectively.

(a) Prechallenge and general observations

Of the three horses, H1 showed a higher base-line total daily clinical score than either of the other two (Figure 6), a feature caused principally by slightly enlarged lymph glands (i.e. a lymph palpation clinical score of 2 as compared to a score of 0 for both H2 and H3) and which may have been due to an underlying chronic respiratory disease. Throughout the trial, the vaccinated pony (H2) exhibited very erratic fibrinogen levels (Figure 9), a feature caused not by infection/inflammation but by fluctuations in physical activity as evidenced by dramatic changes in the levels of two muscle enzymes (aspartate aminotransferase and creatinine kinase; data not shown). It is apparent from a study of individual clinical indices and the total daily clinical score prior to challenge that there were no adverse reactions to the vaccination regimen.

In response to vaccination, there was a significant increase (up to 35-fold) in the titre of serum IgG against FgBP in horse H2, but only a marginal increase in horse H1. As expected the unvaccinated control H3 showed baseline serum IgG titres prior to challenge (Figure 11). Similar profiles were obtained for secretory IgG (Figure 12).

(b) Post-challenge observations

All three horses were challenged on day 61, nineteen days after the final vaccination (day 42). All horses remained stable for at least 11 days post-challenge (day 72). The vaccinated pony (H2) never showed any signs of illness over the full course of the trial. There was no significant increase in clinical scores (Figures 6 and 7) and *S. equi* was never isolated from nasal swabs. Eleven days post-challenge, the vaccinated horse H1 developed an increase in temperature (increase in score from 0 to 4) followed by an increase in nasal discharge (increase in score from 0 to 2; see Figures 6 and 7). Its total daily clinical score increased over two-fold to a maximum score of 13 and, there was an increase in levels of fibrinogen and white blood cells (see Figure 8). That the infection was strangles was confirmed by the isolation of *S. equi* from the nasal discharge. After 11 days, the horse had recovered.

Twenty days post-challenge (day 81), the temperature of unvaccinated control horse, H3, started to increase (Figure 7). This was followed by an increase in throat swelling (increase in score from 0 to 4) and excessive nasal discharge (increase in score from 0 to 4). Significantly, the clinical symptoms of disease of this horse were much more severe than that evidenced in either of the vaccinates. The total daily clinical score of H3 increased seven-fold to a maximum score of 19. Together with obvious clinical signs of illness and distress there was also a clear increase in the levels of fibrinogen and white blood cells (Figure 10), the isolation of *S. equi* from nasal discharges confirming the aetiology of disease. After 9 days of severe infection (day 90), the condition of H3 improved and the horse was fully recovered by day 101 (see Figures 6 and 7).

Neither of the vaccinates showed significant increase in serum IgG to FgBP following challenge. Whereas, there was a marked (up to 10-fold) increase in serum IgG levels during the convalescent period for unvaccinated control H3 (Figure 11). In contrast, all three horses showed some increase in the levels of secretory IgG following challenge (Figure 12).

Three important conclusions can be drawn from this equine trial. Firstly, intranasal vaccination with purified FgBP in PBS does not cause any discernable local or systemic side effects. Secondly and more

significantly, vaccination of horses with purified FgBP, in the absence of adjuvants or transdermal penetrants, affords protection against experimental *S. equi* infection. Thus, the vaccinated pony (H2) did not show any clinical symptoms of disease, whereas the other vaccinated horse (H1) showed a much milder form of the disease than that evidenced by the unvaccinated control horse (H3). Finally, in view of the above, purified FgBP has considerable potential as a constituent of a subunit vaccine against strangles.

Cloning of the gene encoding the FgBP

The general strategy for cloning the gene encoding the FgBP involved: (a) the design of a degenerate oligonucleotide probe made from a knowledge of amino acid sequence; (b) Southern blotting experiments to identify positive clones in *S. equi* DNA libraries; (c) subcloning and sequencing of positive-reacting restriction fragments.

Design of oligonucleotide probe

To facilitate the design of a suitable oligonucleotide, N-terminal amino acid sequence analysis of defined peptides fragments of FgBP was undertaken. Peptide fragments were generated as follows. Mutanolysin-soluble extracts of purified cell-wall material from *S. equi* were electrophoresed on SDS-polyacrylamide gels containing 12.5% [wt/vol] polyacrylamide. The SDS-gels were stained with Coomassie-brilliant blue and bands corresponding to FgBP were excised from the gel. These acrylamide strips, containing about 5-10 ug protein, were then equilibrated in Laemmli running buffer, inserted into wells of a new SDS-gel (12.5% [wt/vol] polyacrylamide) and overlayed with Laemmli sample buffer containing staphylococcal V8 protease. Samples were electrophoresed briefly (20 min, 10 mA) then *in situ* proteolysis allowed to proceed for one hour. Electrophoresis was then continued in the standard fashion. Analysis revealed that FgBP could be degraded to several peptides with apparent M_rs 3,000-14,000. These peptides were transblotted onto Problot™ and N-terminal amino acid sequence analysis was performed directly on three of these peptides using an Applied Biosystems 447A pulsed-liquid protein sequencer. Analysis yielded two distinct sequences. These are given as follows in standard single letter code (1) NSEVSRTATPRL; and (2), LQKAKDERQALTESFNKTLS. A suitable degenerate oligonucleotide (Figure

13) was then made from a consideration of amino acid sequence No. 2 above.

Isolation of S. equi genomic DNA

Genomic DNA was isolated from *S. equi* using a modification of the method described by Lindberg et al. (1972). Bacteria were cultured in 150 ml of broth as described in the section entitled "Isolation of cell wall-associated proteins". Bacteria were then harvested, washed once in 50 mM Tris-malate (pH 7.0) containing 10 mM $MgCl_2$, once in 100 mM Tris-HCl (pH 7.5) containing 10 mM EDTA, 120 mM NaCl and finally resuspended in 6 ml of 100 mM Tris-HCl (pH 7.5) containing 10 mM EDTA, 120 mM NaCl and incubated for 30 min at 37°C with mutanolysin (8,000 U) and lysozyme (50 mg). Isolation of DNA then followed the published procedure (Lindberg et al., 1972).

Alternatively, genomic DNA was isolated from *S equi* essentially as described by Yu and Ferretti (1989) with the following modifications. Prior to harvesting of bacterial cells, the 20-ml bacterial culture was treated for 30 min with hyaluronidase (0.3 µg/ml). After harvesting, washing, and resuspension in 2 x 450-ul volumes of resuspension buffer (10 mM Tris-HCl, 50mM EDTA, pH 8.0), each 450-µl aliquot of cells was treated with 125 U of mutanolysin, 0.5 mg of lysozyme, 50 µg of DNase-free RNase, 250 µg of proteinase K and 1% (wt/vol) sarcosyl using the incubation conditions outlined by Yu and Ferretti (1989). The DNA was then extracted twice with CTAB (hexadecyltrimethyl ammonium bromide)-chloroform as described by Wilson (1995). The resultant DNA was further purified by stepwise extraction with equal volumes of (a) phenol/chloroform (1:1) and (b) chloroform/isoamyl alcohol (24:1), before finally precipitating with 0.6 vol of isopropanol. Ethanol-washed pellets were finally resuspended in TE buffer (10 mM Tris-HCl and 10 mM EDTA, pH8.0).

Construction of an initial gene bank of S. equi DNA in lambda GEM11

A gene bank in the lambda replacement phage, λGEM11, was made essentially as described by the suppliers (Titus, 1991a). *S. equi* genomic DNA was partially cleaved with the restriction endonuclease, *Sau3*A1, in order to generate fragments in the size range 15-20 kb. These were then partially filled-in with dATP and dGTP using DNA polymerase 1 Klenow fragment and were ligated with the left and right arms of the

λGEM11, which had been precleaved with *Xho*I and partially filled-in with dATP and dGTP. Size selection of inserts is achieved during packaging as only arms containing inserts of between 9 and 23 kb are encapsidated. The titre of the resultant library was 2×10^6.

5'-end labelling of degenerate oligonucleotide

The degenerate oligonucleotide (Figure 13) was labelled with ^{32}P dATP using T4 polynucleotide kinase as described by S. Tabor (1995). The reaction mixture containing forward exchange buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM dithiotreitol [DTT] and 0.1 mM spermidine), 10 pmol degenerate oligonucleotide, 50 μCi γ-^{32}P dATP (3000 Ci/mmol^{-1}) and T4 polynucleotide kinase (10 U) was incubated at 37°C for 50 min. The reaction was terminated by addition of 25 mM EDTA (pH 8.0) and the volume of the reaction mixture was increased to 100 μl. Unincorporated dATP was removed from the labelled probe using a Sephadex G-25 column (NAP-5) as described by the supplier (Pharmacia LKB Biotechnology).

Plaque hybridizations

Plaques of the λ library were propagated on *E. coli* LE392 (about 500 plaques per plate; Figure 14) using the overlay method outlined in Titus (1991a). Plaque hybridizations were based on the method of Southern (1975) as outlined by O'Reilly et al. (1988) with the following modifications. Plaque blots were hybridized at 50°C for 2 h in prehybridization solution (5X SSC [saline sodium citrate contains 0.17 M sodium citrate and 0.15 M NaCl], 0.5% [wt/vol] SDS and 5X Denhardts solution) and hybridized at 50°C for 18 h in prehybridization solution containing 1 pmol labelled oligonucleotide probe. Blots were then washed at 37°C for 15 min in 5X SSC, for 15 min in 2X SSC and at 42°C for 15 min in 1X SSC. The blots were then sealed into plastic bags and exposed to X-ray film at -70°C for 3-7 days.

The above hybridization experiments identified one plaque (λSE12.1) which reacted positively with the labelled oligonucleotide. This plaque was picked, resuspended in phage buffer (20 mM Tris-HCl, pH 7.4 containing 0.1 M NaCl and 10 mM $MgSO_4$) and propagated to homogeneity.

Isolation of DNA from λ SE12.1

DNA was isolated from λ SE12.1 by a method which involved purification and concentration of phage particles, lysis of phage capsids and purification of released DNA. Firstly, a high titre plate stock of λ SE12.1 (3×10^{11}) plaque forming units [PFU] were prepared as detailed by O'Toole & Foster (1988). This plate stock was then used to prepare a high titre liquid lysate stock of λ SE12.1 as described by O'Toole & Foster (1988) with the following modifications. 500 µl of an 18-h culture of *E. coli* LE392 grown in L-broth containing 2% (wt/vol) maltose and 10 mM $MgSO_4$, was incubated at 37°C for 15 min with 5×10^8 PFU of λ SE12.1 in the presence of 10 mM $CaCl_2$ and 10 mM $MgCl_2$. This mixture was then added to 250 ml of phage medium (1% casamino acids, 1 x M9 salts, 0.4% (wt/vol) glucose, 0.4% (wt/vol) maltose, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 30 µg/ml tryptophan) in a 2-litre baffled flask and incubated at 37°C for about 6 h at 300 rpm. 5 ml of chloroform was then added to the lysate in order to kill any remaining bacteria. Phage particles were precipitated by addition of NaCl (to a final concentration of 0.5 M) and PEG 6000 (to a final concentration of 10% [wt/vol]) to the lysate and purified to homogeneity on caesium chloride gradients following the procedure outlined by O'Toole & Foster (1988). DNA was isolated from the purified particles as detailed by Maniatis et al. (1982).

Southern hybridization experiments

Phage DNA was digested with various restriction enzymes using conditions described by the manufacturers and the restriction digests were analysed by Southern blotting experiments with the ^{32}P-labelled oligonucleotide probe (Southern, 1975). Prehybridization, hybridization and stringency of washings were performed as described in the section entitled "Plaque hybridizations". The results of the Southern blotting experiments showed that the recombinant phage possessed a *S. equi* DNA insert of about 15 kb. A 1.8 kb *Sac*I restriction fragment which was located adjacent to one of the phage arms reacted with the oligonucleotide probe.

*Cloning of 1.8 kb Sac*I restriction fragment

*Sac*I restriction fragments of λ SE12.1 was ligated to

*Sac*I-digested plasmid pGEM-7 Zf+ (Promega) and transformed into *E. coli* XL1-Blue (Figure 14) as described by Titus (1991b) and Seidman et al., 1995. Transformants were plated onto L-agar plates containing ampicillin (100 µg/ml, 5-bromo-4-chloro-3-indolyl ß-D-galactopyranoside (X-gal; 40 µg/ml) and isopropyl-ß-D-thiogalactopyranoside (IPTG; 40 µM). Plasmid DNA was isolated from several transformants (Birnboim, 1983; Birnboim & Doly, 1979) and analyzed in Southern hybridization experiments using the labelled oligonucleotide probe. One recombinant of pGEM7 containing a positive-reacting 1.8 kb insert was identified and the recombinant plasmid is termed pFBP100.

*DNA sequencing of Sac*I restriction fragment

The nucleotide sequence of most of the *Sac*I insert was determined. This involved preparation of plasmid DNA, generation of nested deletions of the plasmid and DNA sequence analysis of 300-400 bp stretches of the said deletions.

Plasmid DNA was purified from broth-grown *E. coli* (pFBP100) by a modified alkaline lysis method as outlined by Feliciello & Chinali (1993). 10 ug of the purified plasmid DNA was cleaved to completion with two restriction enzymes *Sph*I and *Eco*RI, which generates exonuclease III resistant and exonuclease III sensitive sites, respectively. 200-300 bp nested deletions of the digested plasmid were generated using exonuclease III (Erase-a-Base™ system) as described by the manufacturer (Titus, 1991c). The deleted plasmid DNA was then treated with Klenow DNA polymerase, ligated with T4 DNA ligase and transformed into *E. coli* DH5∝ (Figure 14). Transformed cells were plated onto L-agar containing ampicillin (100 µg/ml). Plasmid DNA was isolated from several of the resultant recombinants from each time point and analysed by restriction digestion and agarose gel electrophoresis.

For DNA sequence analysis, plasmid DNA with overlapping deletions was isolated by the method of Feliciello and Chinali (1993). Automated DNA sequence analysis of the overlapping deletions was performed using an Applied Biosystems 373A DNA sequencer and *Taq* DyeDeoxy™ terminator chemistry (DNA sequencing service, King's College School of Medicine and Dentistry, London). DNA sequence was determined using the M13 forward primer 24mer (5' CGCCAGGGTTTTCCCAGTCACGAC) and, where appropriate, specific

primers synthesized from a knowledge of established DNA sequence.

1400 bp of nucleotide sequence was determined in one strand only (Figure 15). Translation, to amino acids, of the reverse complement of the 1400 bp sequence resulted in the identification of two stretches of amino acid sequence corresponding to those determined from direct amino acid sequence analysis of V8 protease fragments of the protein itself (Figure 15 and Section entitled "Amino acid sequence analysis of FgBP). Immediately preceding one of these amino acid sequences (sequence No. 1) is a stretch of 36 amino acids which shows homology with signal sequences of other streptococcal cell wall proteins. The results of the nucleotide sequencing experiments had thus identified an open reading frame (ORF) encoding the 5' end of the gene (*fbp)* for FgBP. However, the absence of a stop codon or of translated amino acid sequence characteristic of the C-terminal cell wall/membrane anchoring domain of cell wall-associated proteins (see Introduction) indicated that the 3' end of the gene was not represented on the cloned fragment.

Cloning of a 6 kb SphI-SfiI restriction fragment

Experiments were performed to isolate a DNA fragment containing the entire gene encoding the FgBP. This involved digestion of λSE12.1 with various restriction enzymes, analysis of the digests in Southern blotting experiments using the *Sac*I fragment as a probe and cloning of an appropriately sized restriction fragment into a plasmid vector.

The 1.8kb *Sac*I fragment, purified from an agarose gel, was random labelled with α-^{32}P dATP using the Prime-a-gene™ labeling system supplied by Promega (Titus, 1991d). The labelled fragment was used, in Southern blotting experiments, to screen λSE12.1 which had been digested with various restriction enzymes. The basic method of Southern blotting was as described in the Section entitled "Southern blotting experiments" with the following modifications. Prehybridizations and hybridizations were carried out a 65°C. The hydridization solution contained 5ng of labelled probe. After hybridization, blots were washed as follows: twice for 2 min at 65°C in a solution containing 2X SSC and 0.5% (wt/vol) SDS, twice for 15 min at 65°C in a solution containing 2X SSC and 0.1% (wt/vol) SDS and twice for 15 min at 25°C in a solution containing 1X SSC and 0.1% (wt/vol) SDS. The results of the Southern blots showed that a

SphI-SfiI restriction fragment of approximate size 6kb reacted with the labelled gene probe.

This *SphI-SfiI* fragment was cloned into the plasmid pGEM-7 Zf(+) (Promega) as follows. Phage DNA was digested to completion with *SfiI* and *SphI*. Digested phage DNA (200 ng) was then filled-in with dNTPs using DNA polymerase 1 Klenow fragment, ligated to 150 ng of *SmaI*-digested pGEM-7 Zf(+) and transformed into *E. coli* DH5α (see Section entitled "Cloning of *SacI* restriction fragment"). Southern blotting experiments identified a recombinant plasmid (pFBP200) which contained the *SfiI/SphI* restriction fragment (Figure 16). The recombinant *E. coli* strain is termed *E. coli* MM2 and was deposited at the National Collections of Industrial and Marine Bacteria under the Accession Number NCIMB 40807 on 25th June 1996.

DNA sequence analysis of the cloned fragment in pFBP200 revealed it to contain a truncated *fbp* gene encoding the first 488 amino acids of the precursor FgBP (see Figure 18). Cloning the entire *fbp* gene was accomplished using the lambda Zap Express™ system as outlined in the sections below.

Construction of a second gene bank of S. equi DNA in lambda ZAP Express™

Genomic DNA, prepared as described in the section entitled "Isolation of *S. equi* genomic DNA", was partially cleaved with the restriction enzyme *Sau3A*, in order to generate fragments in the size range 10-12kb. The digested DNA was ligated to λZAP Express™ vector arms (*BamHI*-CIAP-treated) and packaged using Gigapack™ III Gold packaging extract according to the manufacturers instructions (Stratagene). The titre of the resultant library was 6 x 10^5pfu/ml.

Digoxigenin (DIG) labelling of the 1.8 kb SacI fragment

Plasmid pFBP100 was digested with *SacI* and the 1.8kb fragment gel purified using the Wizard™ PCR preps DNA purification system. The purified fragment was labelled with DIG by random priming using Klenow enzyme as outlined by the manufacturer (Boehringer Mannheim).

Screening of the second gene bank of S. equi

Plaques (approximately 15,000) from the λZAP Express™ library were propagated on *E. coli* XL1-Blue MRF' using the overlay method outlined by Titus (1991a). Plaque hybridizations were carried out as outlined in 'The DIG system user's guide for filter hybridization' (Boehringer Mannheim). Hybridizations were performed at 68°C in standard hybridization buffer (5 x SSC, 1.0% [wt/vol] Boehringer blocking reagent, 0.1% [wt/vol] N-lauroylsarcosine, 0.2% [wt/vol] SDS). Chemiluminescent detection was carried out using anti-DIG-alkaline phosphatase and the alkaline phosphatase substrate, CSPD™.

Isolation of positive subclones from the λZAP Express™ library

Several positive reactive plaques were identified in the above gene library and were propagated to homogeneity. The pBK-CMV phagemid vectors, containing the cloned inserts, were excised from the ZAP Express™ vector using Exassist™ interference-resistance helper phage according to the manufacturer's instructions (Stratagene). Phagemid DNA was isolated from the *E. coli* XLOLR transformants as described by Feliciello and Chinali (1993). One clone (termed pFBP700), which contained an insert of about 3800 bp (see Figure 17 for partial restriction map), was chosen for further study. The recombinant *E. coli* strain XLOLR harbouring pFBP700 is termed *E. coli* MM20 and was deposited at the NCIMB under the Accession No. 40883.

DNA sequencing

DNA sequencing (Figure 18) of the bulk of the 3800 bp insert was determined. The sequencing strategy used was similar to that described in the Section entitled "DNA sequencing of *Sac*I restriction fragment", and involved the generation of subclones containing overlapping deletions suitable for double-stranded sequencing using a combination of universal M13 primers and specific synthetic primers.

Nucleotide and amino acid sequence analysis of fbp

The FgBP is encoded by an ORF of 1,605 bp which is preceded by sequences typical of promoter signals and ribosome-binding sites (Fig.

18). The ORF itself has the following features. The first 36 residues of the deduced amino acid sequence show features characteristic of a signal sequence. This region is homologous to signal sequences of other cell wall proteins (see Goward et al., 1993). Thus, the *fbp* gene encodes for a primary translation production of 534 amino acids (M_r58,352) which is post-translationally processed to yield a putative mature protein of 498 amino acids (M_r54,604).

The sequence of the mature protein contains features characteristic of cell wall-associated proteins (see Introduction). Firstly, the sequence contains two blocks (A and B) of repeated sequences. The A repeats are 21 amino acid in length and are repeated at least twice. The B repeats are about 11 amino acids long and are repeated twice. Secondly, the C-terminal part of the protein contains a putative wall/membrane anchoring domain. Thus, the consensus LPSTGE motif is followed almost directly by 21 hydrophobic amino acids (membrane spanning region) and a short charged tail of five amino acid residues (see Figure 18). Structural predictions (Lupas, 1996; Lupas et al., 1991; Berger et al., 1995, Wolf et al [World Wide Web at http://theory.lcs.mit.edu/multicoil]) strongly suggest that at least 60% of the FgBP exists as a helical coiled-coil dimer like M-proteins of *S. pyogenes*.

Computer assisted searches of existing databases (Altschul et al., 1990) revealed that the FgBP showed no significant homology, at the nucleotide and amino acid level (except in the signal sequence and wall/membrane spanning regions), to any other submitted sequence. Nor did the sequence of the *fgb* gene show any homology to the partial sequence published by Timoney and co-workers (Galán & Timoney, 1987, Galán et al., 1988; Timoney et al., 1991) for their M-protein from *S. equi*.

Expression of a truncated FgBP

Experiments were conducted to assess the feasibility of expressing the FgBP from a *fbp* fragment cloned into a heterologous expression system. Accordingly, a *fbp* fragment encoding a truncated form of the FgBP (FgBP lacking its C-terminal anchor region) was cloned into *E. coli* using the QIAexpress pQE30 vector system (Qiagen GmbH, Germany). This vector places a hexahistidine affinity tag at the N-terminus of the resultant fusion protein whose expression is under the control of the *lac* promoter and

thus subject to induction by lactose or appropriate analogue e.g. isopropyl ß-D-thiogalactoside (IPTG).

Cloning of truncated fbp gene into the pQE expression vector

The region of *fbp* encoding the first 434 amino acids of the mature protein (i.e. amino acids 37-470 of the precursor protein, see Figure 18) was cloned in the correct reading frame into pQE30. The *fbp* fragment of DNA was first amplified by polymerase chain reaction (PCR) using *pfu* polymerase (Stratagene) and appropriate forward and reverse primers. The resultant PCR product was then gel purified, ligated to restriction-digested pQE30 and transformed into *E. coli* XL1-Blue (Titus, 1991b and Seidman et al., 1995). Positive transformants were identified by restriction digestion of isolated plasmid DNA and confirmed by PCR analysis using the above primers. SDS-PAGE analysis of cell lysates confirmed that all transformants expressed two high molecular weight proteins (apparent M_rs of approximately 220,000 and 90,000) which were not present in lysates of *E. coli* XL-1 Blue harbouring pQE30 alone. Of these, the higher M_r species was present at higher concentrations (see Figure 19). Both proteins reacted with rabbit anti-FgBP antibodies in Western immunoblots.

Purification of the FgBP truncate

The hexahistidyl-tagged FgBP truncate was purified using metal chelate affinity chromatography using methodology described by Qiagen GmbH, Germany. Briefly, transformants were grown overnight at 37°C in 10 ml of 2xYT+G medium (tryptone[16 g/l], yeast extract [10 g/l], NaCl [5 g/l], glucose [0.2 g/l] containing ampicillin (100 µg/ml). The overnight culture was then used to inoculate 500 ml of fresh medium and the culture grown to an A_{600nm} of 0.9. IPTG was then added to a final concentration of 1 mM and growth was continued for a further 3 hours. Bacterial cells were harvested, washed and resuspended in 30 ml binding buffer (20 mM Tris-HCl pH 7.9, 0.5M NaCl) containing RNase (50 µg/ml). DNase (50 µg/ml), and the two protease inhibitors, phenylmethylsulfonyl-floride (2 mM) and benzamidine-HCl (2 mM). Cells were lysed by passage through a French pressure cell (32,000 p.s.i.) and the lysate was cleared by centrifugation (45,000 x g, 1 hour, 4°C) to yield a soluble supernatant fraction containing the expressed proteins. This soluble fraction was applied to a 1 ml nickel-iminodiacetic acid (IDA) Sepharose column which was then washed

extensively with binding buffer until the OD_{280nm} reached zero. Hexahistidyl-tagged FgBP truncates were recovered using a 0-1M gradient of imidazole in binding buffer and found to elute maximally at 100-200 mM imidazole. A fibrinogen-affinity blotting assay revealed that the truncated proteins (apparent M_rs 220,000 and 90,000) bound horse fibrinogen in a manner analogous to native FgBP expressed by *S. equi* (Fig. 19).

The following conclusions can be drawn from these experiments: (i) A *S. equi* fibrinogen binding protein, lacking its C-terminal anchor, can be expressed from a *fbp* fragment cloned into a heterologous expression system; (ii) The resultant FgBP truncate (M_r50,270) behaves during SDS-PAGE analysis in a manner analogous to the native FgBP (M_r54,604). In both cases, the dominant species migrates with an apparent M_r of approximately 220,000. Structural predictions based on amino acid sequence strongly suggest that the apparent M_r of 220,000 reflects the propensity of the native and recombinant proteins to form alpha-helical coiled-coil multimeric structures (see section entitled "Nucleotide and amino acid sequence analysis of *fbp*"). (iii) Removal of 64 amino acids from the C-terminus of FgBP does not significantly affect the ability of the protein to bind fibrinogen; (iv) The above provides an example of a method of producing a *S. equi* fibrinogen-binding protein (or suitable truncate) from a genetically-engineered host cell containing the *fbp* (or fragment thereof).

Suppliers of reagents and chemicals

CnBr-activated Sepharose™ was obtained from Pharmacia LKB Biotechnology, Milton Keyes, U.K. ImmunoPure™Immobilized protein G was obtained from Pierce Chemical Co., IL, USA. 96-well ELISA plates were obtained from Sarsdedt Ltd, Wexford, Ireland. Horse fibrinogen, horseradish peroxidase, peroxidase-labelled affinity-purified anti-mouse IgG (H+L), and iminodiacetic acid Sepharose were obtained from Sigma Chemical Co., Dorset, U.K. Restriction enzymes and other molecular biology reagents were obtained from Promega Corporation, WI, USA and New England Biolabs Inc., MA, USA. The pGEM-7 Zf(+) vector, Erase-a-Base™, Prime-a-gene™ labeling system and Wizard™ PCR preps DNA purification system were obtained from Promega Corporation, WI, USA. Peroxidase-labelled affinity-purified goat anti-horse IgG (H+L) was

obtained from ICN Biomedicals Inc., CA, USA. Peroxidase-labelled affinity-purified goat anti-horse IgA (alpha-chain specific) was obtained from Bethyl Laboratories Inc., TX. MPL+S-TDCM Ribi adjuvant was obtained from RIBI Immunochem Research Inc., MT, USA. The DIG labelling and detection kits were obtained from Boehringer Mannheim, GmbH, Biochemica. The ZAP Express™ predigested vector (BamHI/CIAP-treated) and Gigapack™ III packaging extract were obtained from Strategene. The QIA express pQE30 vector system was obtained from Qiagen GmbH, Germany.

Standard Amino Acid Abbreviations

Amino acid	Single letter code
Alanine	A
Arginine	R
Asparagine	N
Aspartic acid	D
Cysteine	C
Glutamine	Q
Glutamic acid	E
Glycine	G
Histidine	H
Isoleucine	I
Leucine	L
Lysine	K
Methionine	M
Phenyalanine	F
Proline	P
Serine	S
Threonine	T
Tryptophan	W
Tyrosine	Y
Valine	V

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CLAIMS

1. A fibrinogen-binding protein isolated from *S. equi* subsp. *equi* having the following characteristics:-

(1) it migrates on Laemmli SDS-PAGE gels with an apparent molecular weight of approximately 220,000,

(2) it binds horse fibrinogen, and

(3) it protects against *S. equi* infection in horses (Strangles), or a truncate thereof.

2. A fibrinogen-binding protein as claimed in Claim 1 characterised in that it is associated with the cell wall of *S. equi*.

3. A fibrinogen-binding protein as claimed in Claim 1 or Claim 2 comprising a multimeric alpha-helical, coiled-coil structure, the individual monomers having a molecular weight of about 55,000.

4. A fibrinogen-binding protein as claimed in any preceding claim comprising an amino-acid sequence selected from:-

(1) NSEVSRTATPRL; and

(2) LQKAKDERQALTESFNKTLS.

5. A fibrinogen-binding protein as claimed in any preceding claim comprising an amino-acid sequence selected from those shown in Figures 15 and 18.

6. A DNA fragment selected from those deposited at The National Collections of Industrial and Marine Bacteria under the Accession Numbers NCIMB 40807 and NCIMB 40883, encoding the *S. equi* fibrinogen-binding protein or a fragment substantially similar thereto also encoding *S. equi* fibrinogen-binding protein and/or Strangles protective activity.

7. A DNA fragment comprising a nucleotide sequence selected from those shown in Figures 15 or 18.

8. A host cell comprising a DNA fragment as claimed in Claim 6 or 7.

9. A method of producing a fibrinogen-binding protein comprising culturing a host cell as claimed in Claim 8 and isolating the protein from the culture.

10. A method of producing an *S. equi* fibrinogen-binding protein comprising the steps:-

(1) genetically engineering a host cell containing the DNA fragment of Claim 6 or 7 to overexpress and/or secrete the fibrinogen-binding protein (or suitable truncate) into the supernatant,

(2) isolating the fibrinogen-binding protein or truncate in a cell-free supernatant fraction, and

(3) purifying the fibrinogen-binding protein or truncate.

11. A method as claimed in claim 10 wherein the fibrinogen-binding protein or truncate is isolated in a cell-free supernatant fraction either by (a) lysis of the host cell followed by centrifugation or by any of a number of filtration methods known in the art or (b) isolation of culture supernatant by centrifugation or by any of a number of filtration methods known in the art.

12. A method as claimed in claim 11 wherein the fibrinogen-binding protein or truncate is purified by fibrinogen-affinity chromatography or other chromatographic procedures known in the art.

13. A vaccine comprising a fibrinogen-binding protein or truncate as claimed in Claims 1 to 5 or as produced by a method as claimed in Claims 10 to 12, or a DNA fragment as claimed in Claim 6 or 7.

14. *S. equi* fibrinogen-binding protein as claimed in any of Claims 1 to 5 for use in the preparation of a vaccine against Strangles infection in horses.

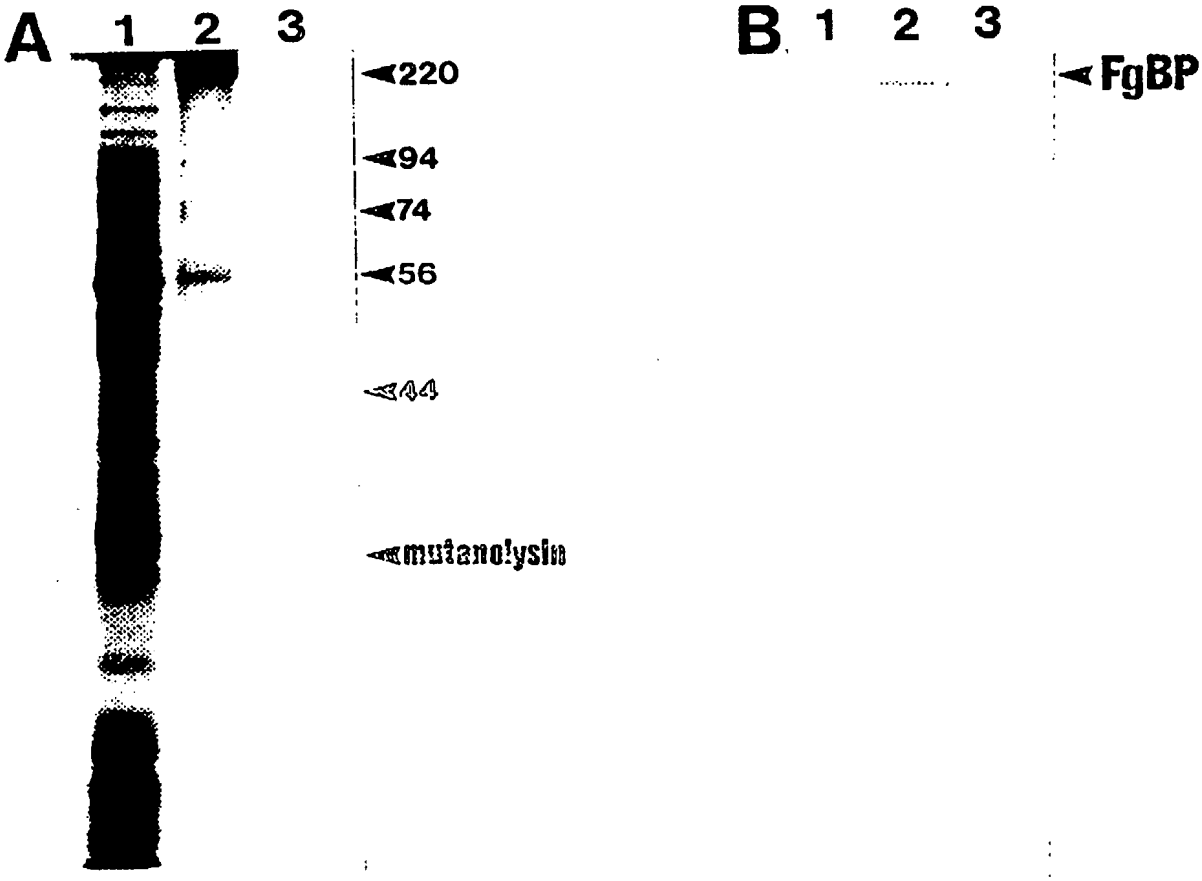


FIGURE 1

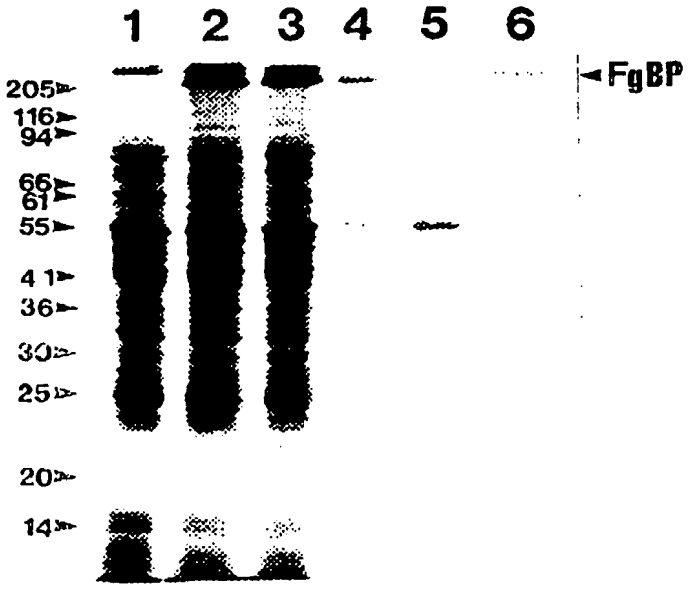
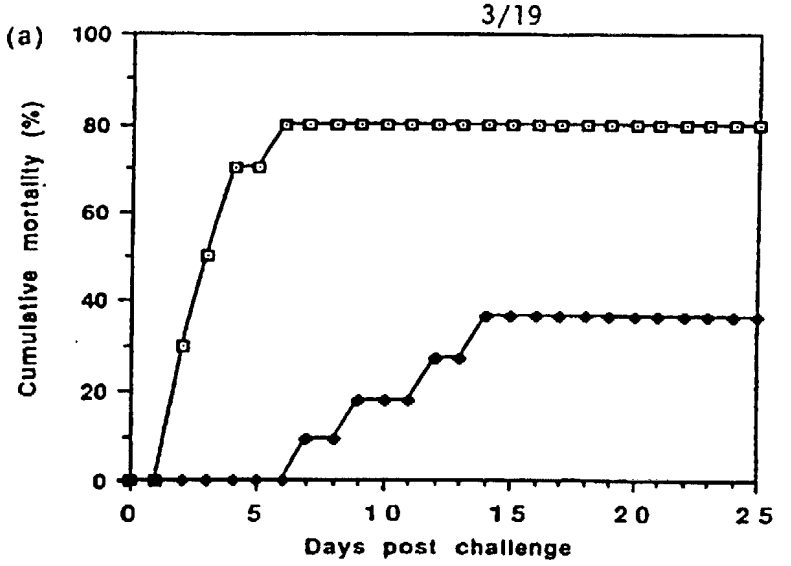


FIGURE 2


(a)
100
80
60
40
20
0
Cumulative mortality (%)
0
5
10
15
20
25
Days post challenge

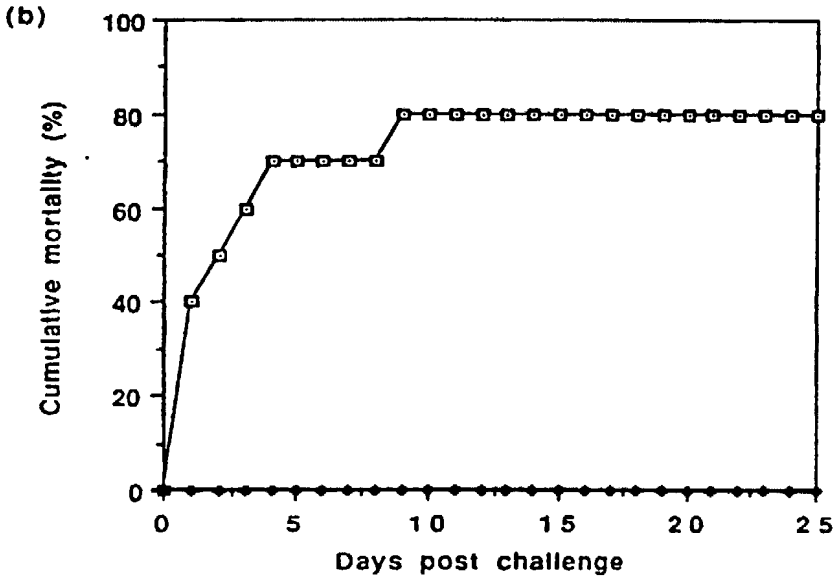

(b)
100
80
60
40
20
0
Cumulative mortality (%)
0
5
10
15
20
25
Days post challenge

FIGURE 3

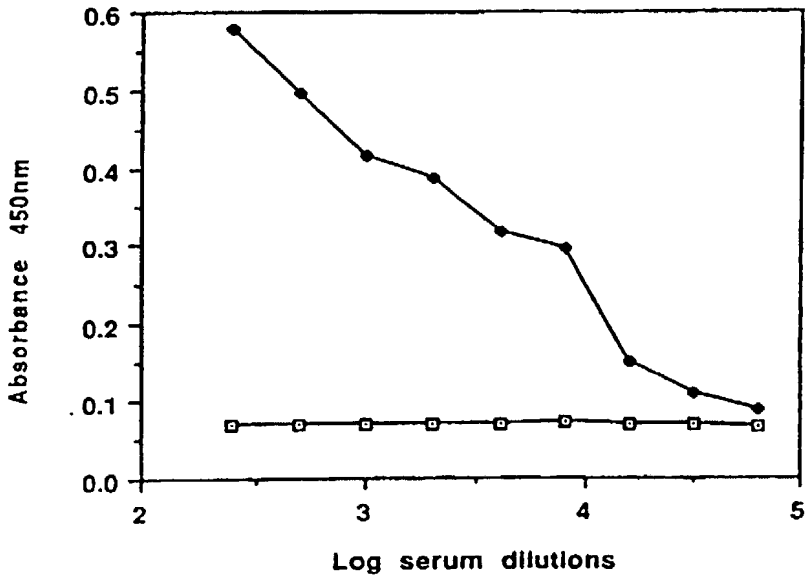

0.6
0.5
0.4
0.3
0.2
0.1
0.0
Absorbance 450nm
2
3
4
5
Log serum dilutions

FIGURE 4

FIGURE 5 Numerical clinical scoring system

general impression	0=normal 1=less active 2=slightly depressed 4=depressed 6=severely depressed	
anorexia	0=normal 4=loss of apetite	
temperature	0=≤38.5 1=38.6-39.0 2=39.1-39.5 3=39.6-40.0 4=40.1-40.5 5=40.6-41.0 6=>41.0	
nasal discharge	0=absent 1=serous 2=mucopurulent 3=purulent	if excessive, 1 point extra
ocular discharge	0=absent 1=serous 2=mucopurulent 3=purulent	if excessive, 1 point extra
throat swelling	0=absent 2=slight 4=moderate 6=severe	
cardiac rate	0=≤35 1=36-45 2=46-55 3=56-65 4=>65	
respiratory rate	0=0-5 1=6-14 2=15-24	
respiratory type	0=costo-abdominal 2=slightly abdominal 4=abdominal 6=strongly abdominal	
stridor	0=absent 2=slight 4=moderate 6=severe	
auscultation	0=normal 2=slight rales 4=dry or wet rales 6=area with no sound	
lymph palpation	0=normal 2=slightly enlarged 4=moderately enlarged 6=strongly enlarged or rupured	if also painful 1 point extra

Figure 5 (contd) Numerical clinical scoring system

palp. larynx	0=no coughing 1=one or two coughs 3=several coughs	if painful 1 point extra
palp. trachea	0=no coughing 1=one or two coughs 3=several coughs	if painful 1 point extra
spontaneous cough	0=absent 2=after inspection 4=before inspection	
lameness	0=absent 3=detectable walk 6=drags or jumps to carry lame leg	for each swollen joint independent of lameness, 3 points extra

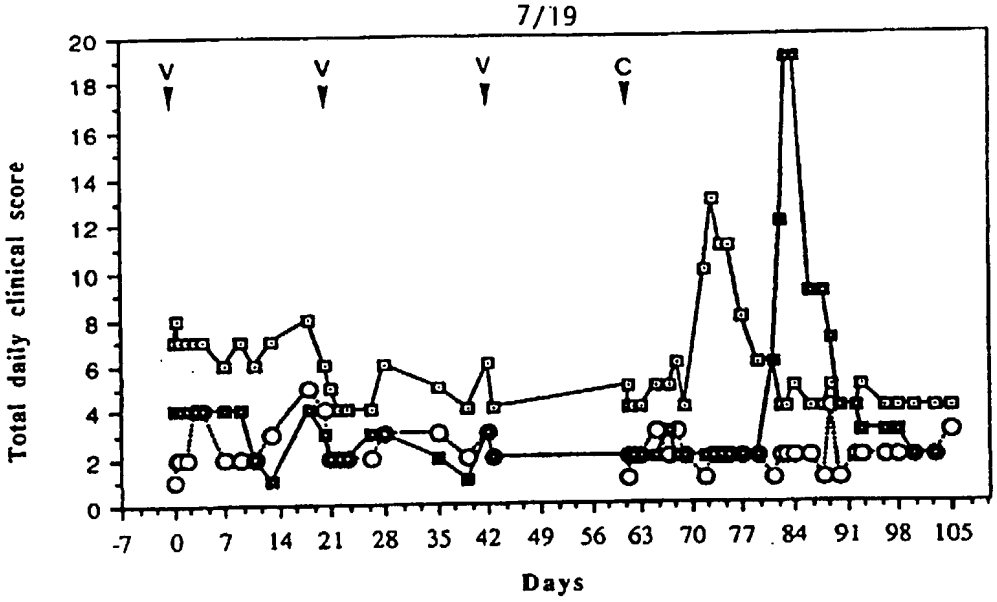


FIGURE 6

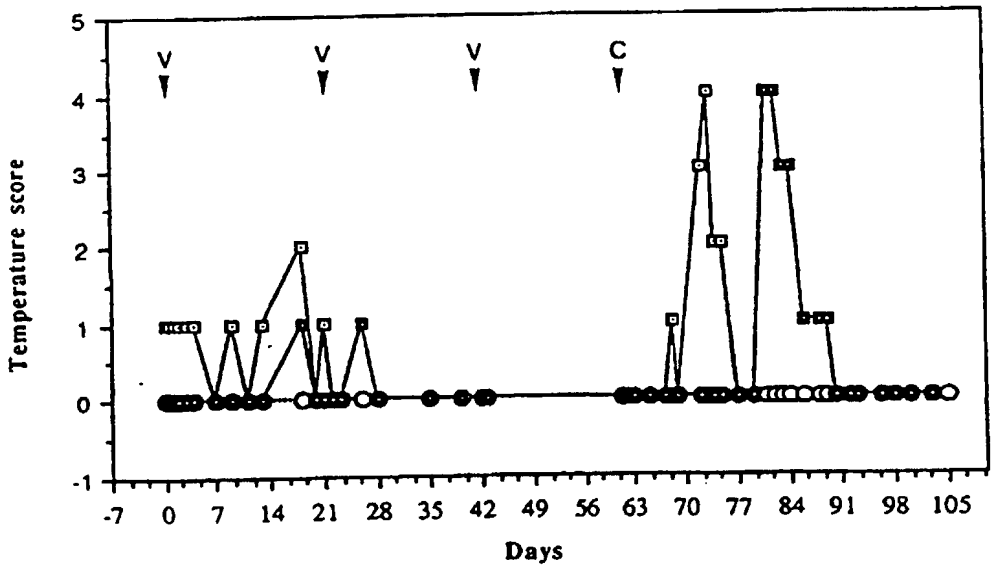


FIGURE 7

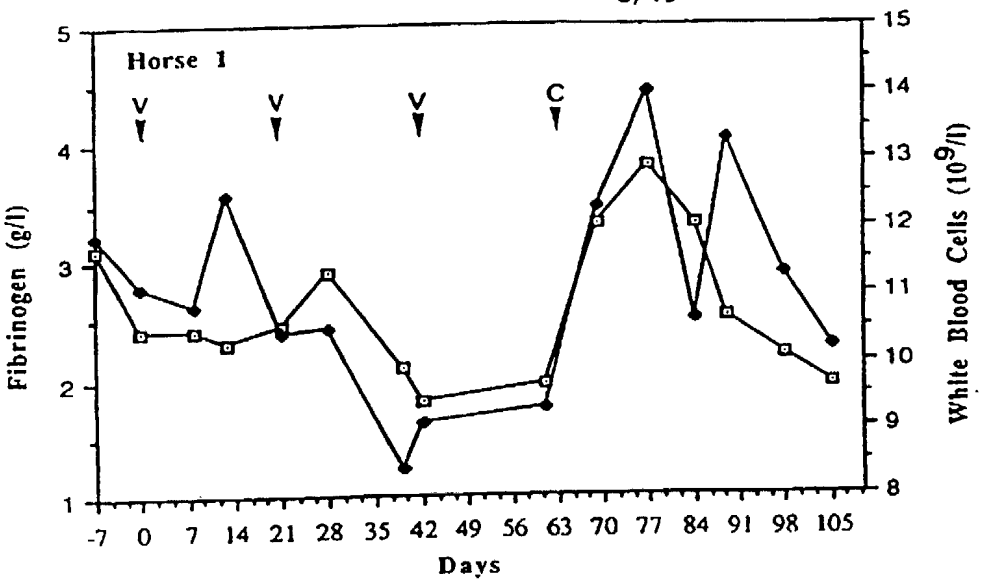


FIGURE 8

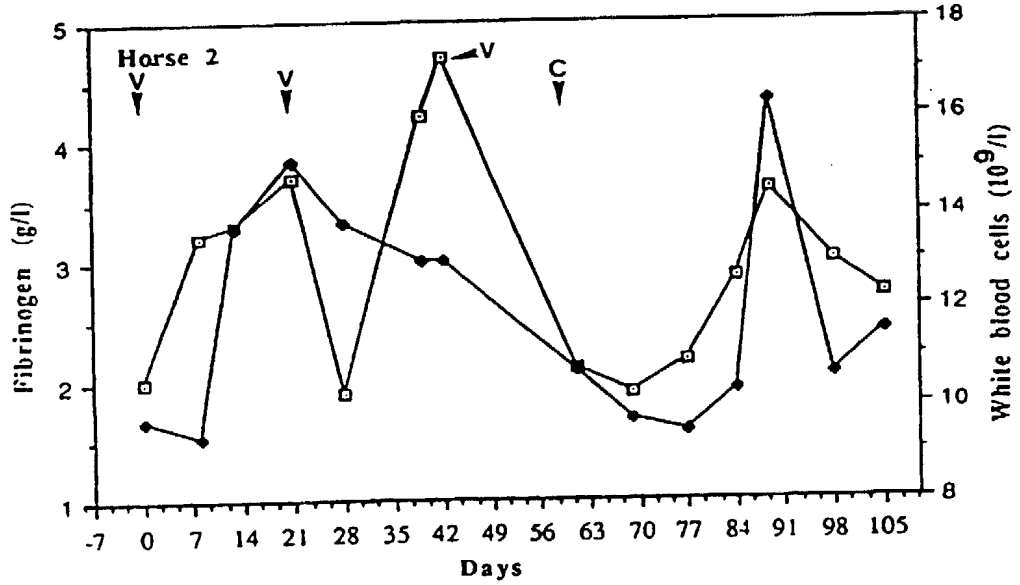


FIGURE 9

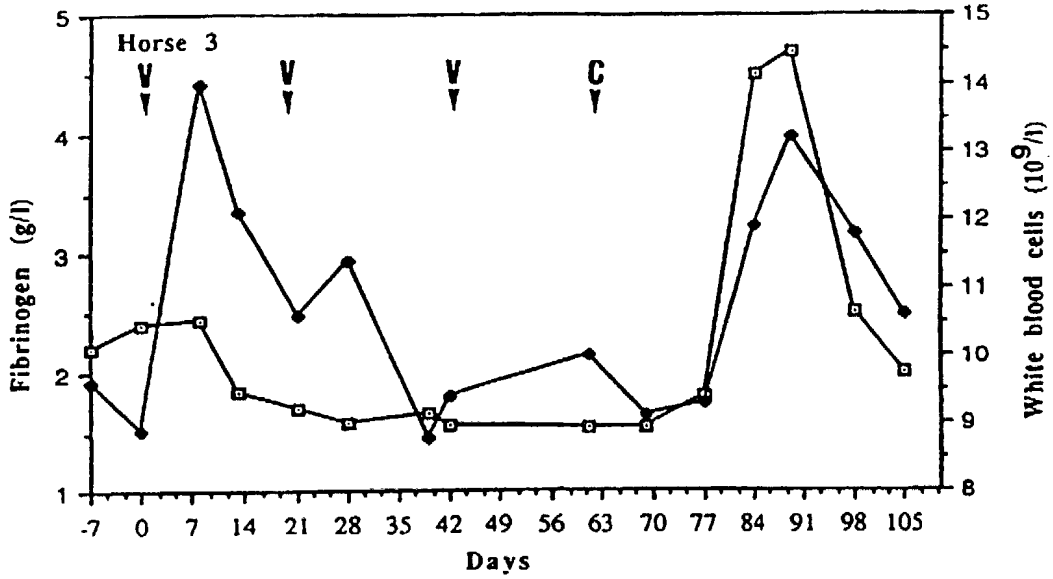

Horse 3
V
V
V
C
Fibrinogen (g/l)
White blood cells (10^9/l)
Days
-7 0 7 14 21 28 35 42 49 56 63 70 77 84 91 98 105
1 2 3 4 5
8 9 10 11 12 13 14 15

FIGURE 10

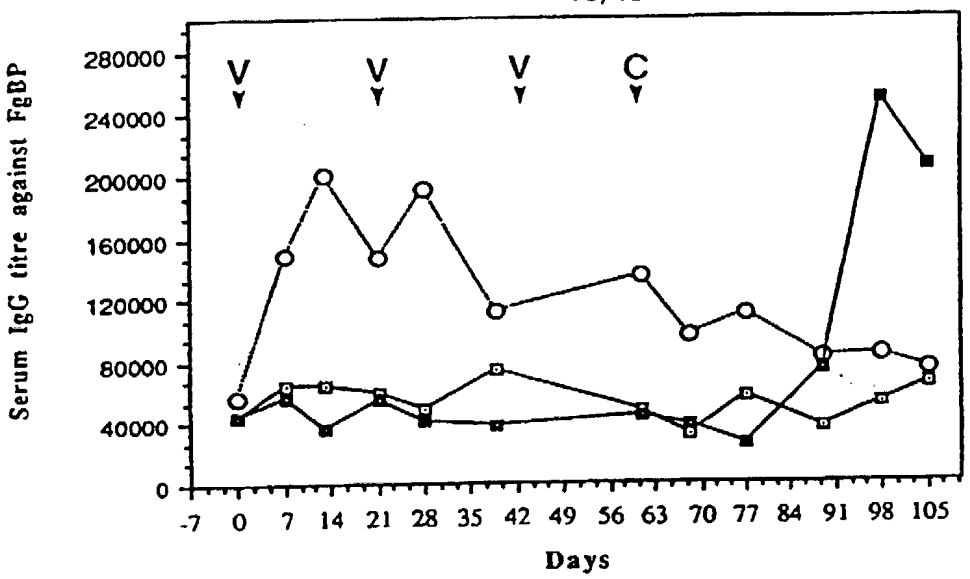


FIGURE 11

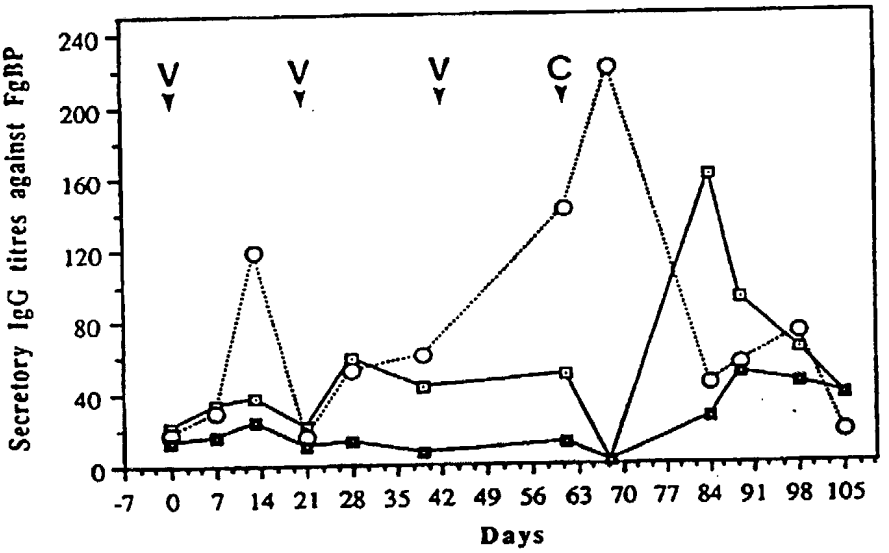


FIGURE 12

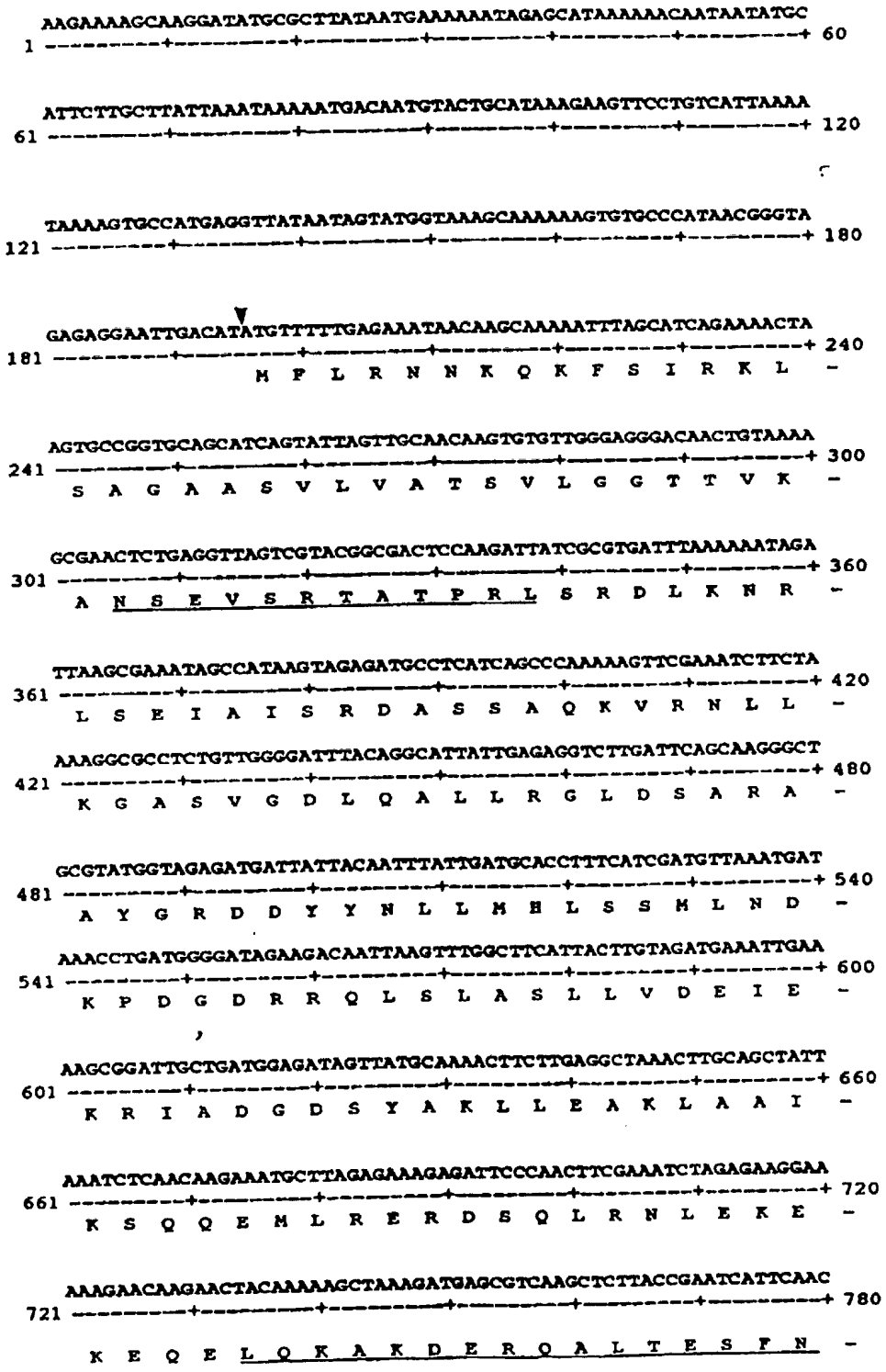
Q	K	A	K	D	E	R
CAA G	AAA G G T	GCA C	AAA G	GAC T	GAA G	CG

FIGURE 13

Figure 14. Genotype of bacterial strains.

Strain	Genotype	Source
E. coli LE392	*hsd R574*, (r_K^-, m_K^+), *supE44*, *sup F58*, *lacY1* or Δ(*lacIZY*)6,*galK2*, *galT22*, *metB1*, *trpR55*	Promega Corporation, WI, USA
E. coli DH5α	ϕ80d*lacZ*ΔM15, *recA1*, *endA1*, *gyrA96*, *thi*-1, *hsdR17* (r_K^-, m_K^+), *supE44*, *relA1*, *deoR*, Δ(*lacZYA-argF*)U169	Life Technologies Ltd., Scotland, U.K.
E. coli XL1-Blue	F' ::Tn*10*, *proA*$^+$*B*$^+$, *lacI*^{q}, Δ(*lacZ*)*M15*/*recA*1 *endA1*, *gyrA96*(Nal^r), thi, *hsdR17* (r_K^-, m_K^+), *supE44*, *relA1*, *lac*	Stratagene Ltd, Cambridge, UK
E. coli XL1-Blue MRF'	Δ(*mcrA*)183Δ(*mcrCB-hsdSMR-mrr*)173, *endA1*, *supE44*, *thi-1*, *recA1*, *gyrA96*, *relA1*, *lac*[F' *proAB lacI*^{q}ZΔ*M15* Tn*10*(Tet^r)]	Stratagene Ltd, Cambridge, UK
E. coli XLOLR	Δ(*mcrA*)*183* Δ(*McrCB-hsdSMR-mrr*)*173 endA1* *thi-1*, *recA1*, *gyrA96*, *relA1*, *lac*[F'*proAB* *lacI*^{q}ZΔ*M15*Tn*10*(Tet^r)], Su$^-$, λ^r	Stratagene Ltd, Cambridge, UK

FIGURE 15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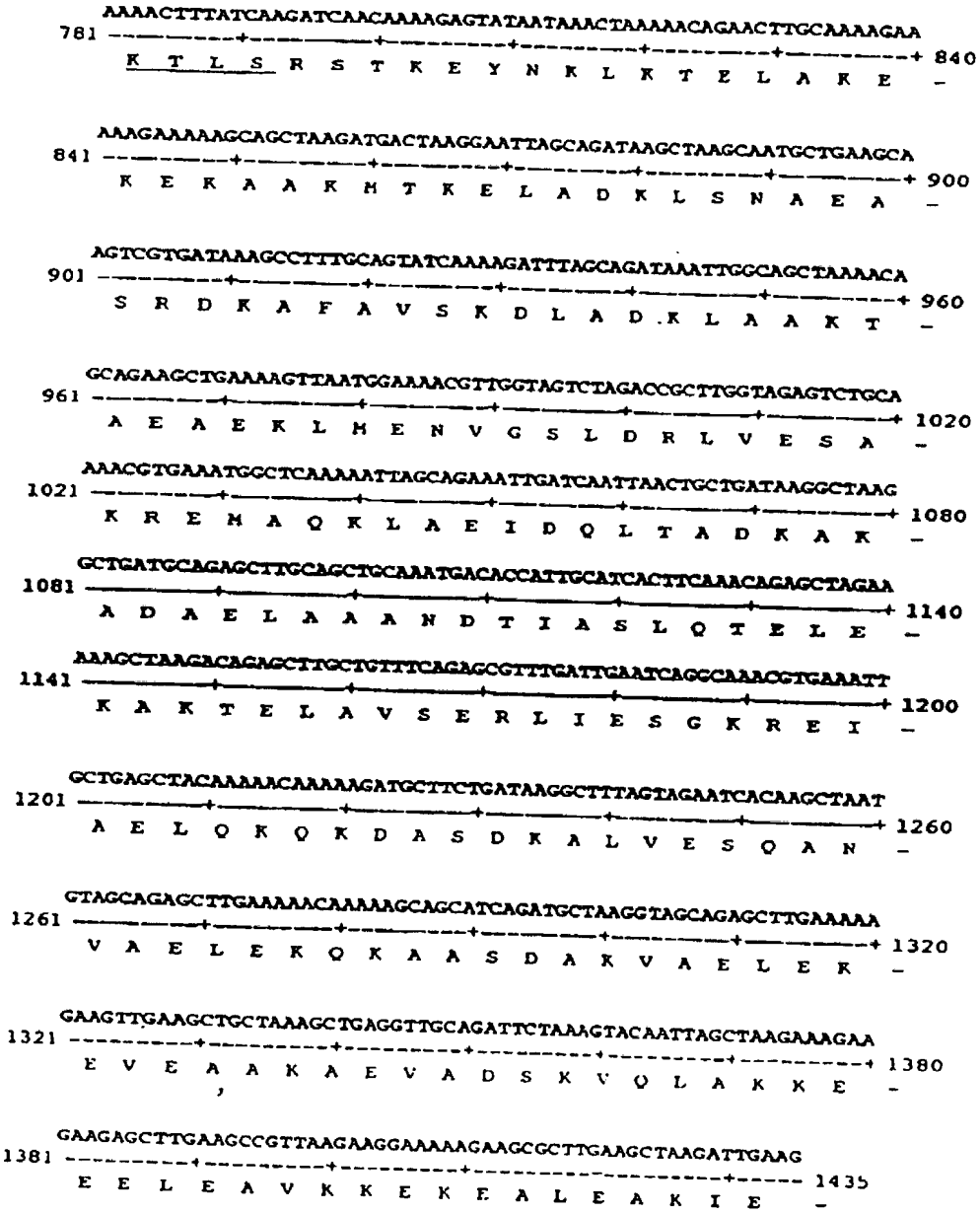
61 120
TAAAAGTGCCATGAGGTTATAATAGTATGGTAAAGCAAAAAAGTGTGCCCATAACGGGTA
121 180
GAGAGGAATTGACATATGTTTTTGAGAAATAACAAGCAAAAATTTAGCATCAGAAAACTA
181 240
M F L R N N K Q K F S I R K L -
AGTGCCGGTGCAGCATCAGTATTAGTTGCAACAAGTGTGTTGGGAGGGACAACTGTAAAA
241 300
S A G A A S V L V A T S V L G G T T V K -
GCGAACTCTGAGGTTAGTCGTACGGCGACTCCAAGATTATCGCGTGATTTAAAAAATAGA
301 360
A N S E V S R T A T P R L S R D L K N R -
TTAAGCGAAATAGCCATAAGTAGAGATGCCTCATCAGCCCAAAAAGTTCGAAATCTTCTA
361 420
L S E I A I S R D A S S A Q K V R N L L -
AAAGGCGCCTCTGTTGGGGATTTACAGGCATTATTGAGAGGTCTTGATTCAGCAAGGGCT
421 480
K G A S V G D L Q A L L R G L D S A R A -
GCGTATGGTAGAGATGATTATTACAATTTATTGATGCACCTTTCATCGATGTTAAATGAT
481 540
A Y G R D D Y Y N L L M H L S S M L N D -
AAACCTGATGGGGATAGAAGACAATTAAGTTTGGCTTCATTACTTGTAGATGAAATTGAA
541 600
K P D G D R R Q L S L A S L L V D E I E -
AAGCGGATTGCTGATGGAGATAGTTATGCAAAACTTCTTGAGGCTAAACTTGCAGCTATT
601 660
K R I A D G D S Y A K L L E A K L A A I -
AAATCTCAACAAGAAATGCTTAGAGAAAGAGATTCCCAACTTCGAAATCTAGAGAAGGAA
661 720
K S Q Q E M L R E R D S Q L R N L E K E -
AAAGAACAAGAACTACAAAAAGCTAAAGATGAGCGTCAAGCTCTTACCGAATCATTCAAC
721 780
K E Q E L Q K A K D E R Q A L T E S F N -

FIGURE 15
CONTINUED



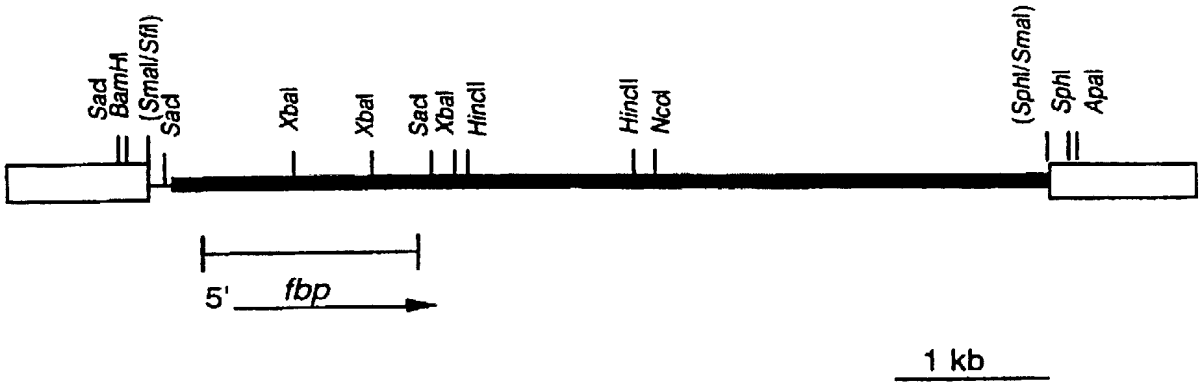

SacI
BamHI
(SmaI/SfiI)
SacI
XbaI
XbaI
SacI
XbaI
HincII
HincII
NcoI
(SphI/SmaI)
SphI
ApaI
5'
fbp
1 kb

FIGURE 16

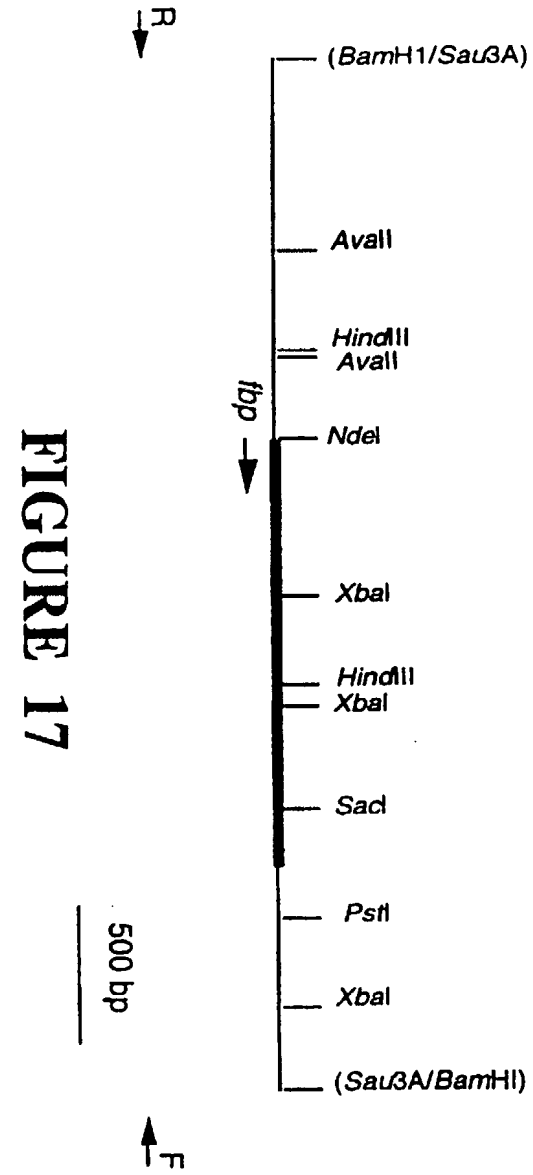
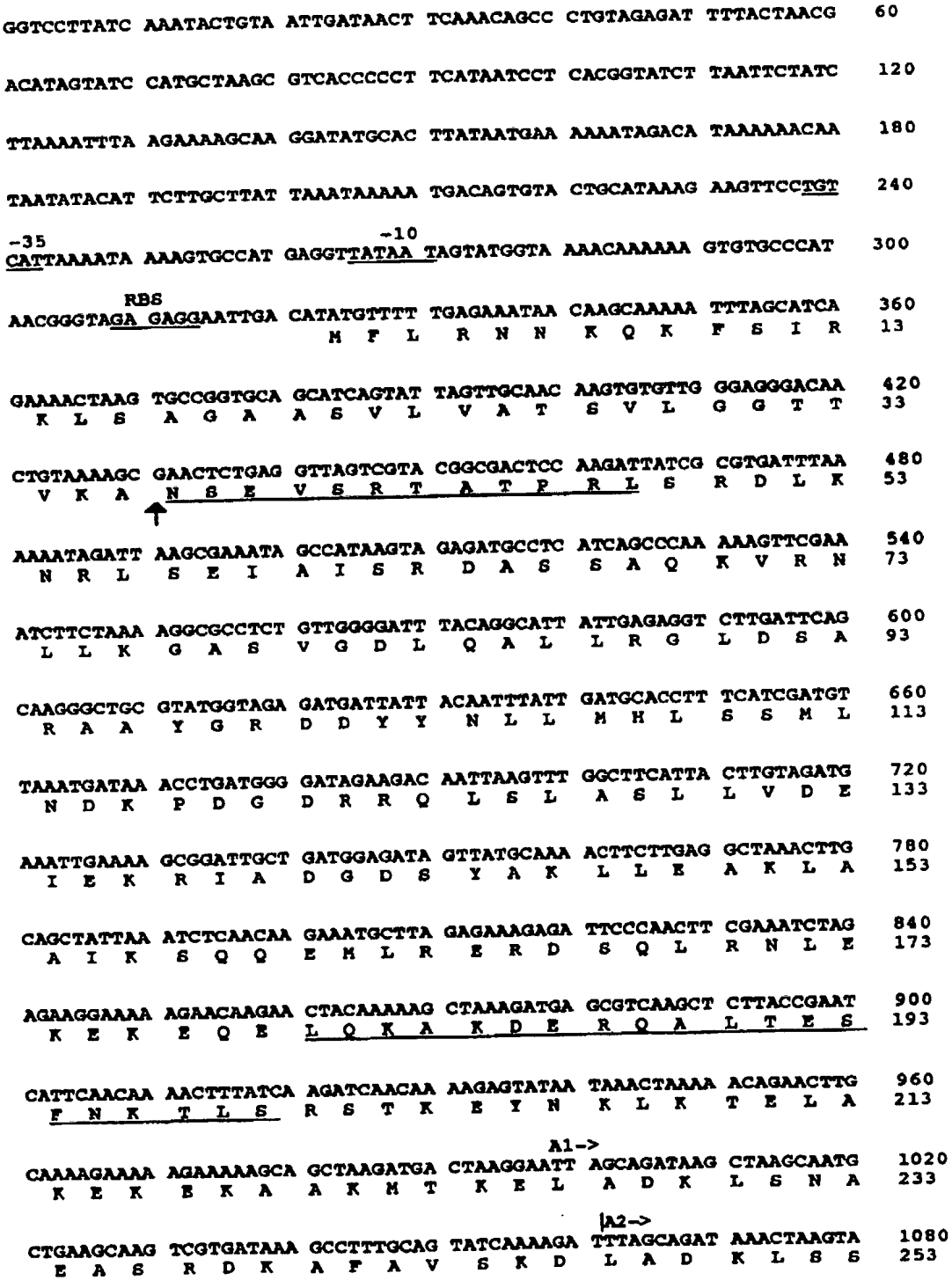

R
(BamH1/Sau3A)
AvaII
HindIII
AvaII
dgl
NdeI
XbaI
HindIII
XbaI
SacI
PstI
XbaI
(Sau3A/BamHI)
500 bp
L

FIGURE 17

FIGURE 18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TTAAAATTTA AGAAAAGCAA GGATATGCAC TTATAATGAA AAAATAGACA TAAAAAACAA 180
TAATATACAT TCTTGCTTAT TAAATAAAAA TGACAGTGTA CTGCATAAAG AAGTTCCTGT 240
-35 -10
CATTAAAATA AAAGTGCCAT GAGGTTATAA TAGTATGGTA AAACAAAAAA GTGTGCCCAT 300
RBS
AACGGGTAGA GAGGAATTGA CATATGTTTT TGAGAAATAA CAAGCAAAAA TTTAGCATCA 360
M F L R N N K Q K F S I R 13
GAAAACTAAG TGCCGGTGCA GCATCAGTAT TAGTTGCAAC AAGTGTGTTG GGAGGGACAA 420
K L S A G A A S V L V A T S V L G G T T 33
CTGTAAAAGC GAACTCTGAG GTTAGTCGTA CGGCGACTCC AAGATTATCG CGTGATTTAA 480
V K A N S E V S R T A T P R L S R D L K 53
AAAATAGATT AAGCGAAATA GCCATAAGTA GAGATGCCTC ATCAGCCCAA AAAGTTCGAA 540
N R L S E I A I S R D A S S A Q K V R N 73
ATCTTCTAAA AGGCGCCTCT GTTGGGGATT TACAGGCATT ATTGAGAGGT CTTGATTCAG 600
L L K G A S V G D L Q A L L R G L D S A 93
CAAGGGCTGC GTATGGTAGA GATGATTATT ACAATTTATT GATGCACCTT TCATCGATGT 660
R A A Y G R D D Y Y N L L M H L S S M L 113
TAAATGATAA ACCTGATGGG GATAGAAGAC AATTAAGTTT GGCTTCATTA CTTGTAGATG 720
N D K P D G D R R Q L S L A S L L V D E 133
AAATTGAAAA GCGGATTGCT GATGGAGATA GTTATGCAAA ACTTCTTGAG GCTAAACTTG 780
I E K R I A D G D S Y A K L L E A K L A 153
CAGCTATTAA ATCTCAACAA GAAATGCTTA GAGAAAGAGA TTCCCAACTT CGAAATCTAG 840
A I K S Q Q E M L R E R D S Q L R N L E 173
AGAAGGAAAA AGAACAAGAA CTACAAAAAG CTAAAGATGA GCGTCAAGCT CTTACCGAAT 900
K E K E Q E L Q K A K D E R Q A L T E S 193
CATTCAACAA AACTTTATCA AGATCAACAA AAGAGTATAA TAAACTAAAA ACAGAACTTG 960
F N K T L S R S T K E Y N K L K T E L A 213
A1->
CAAAAGAAAA AGAAAAAGCA GCTAAGATGA CTAAGGAATT AGCAGATAAG CTAAGCAATG 1020
K E K E K A A K M T K E L A D K L S N A 233
|A2->
CTGAAGCAAG TCGTGATAAA GCCTTTGCAG TATCAAAAGA TTTAGCAGAT AAACTAAGTA 1080
E A S R D K A F A V S K D L A D K L S S 253

FIGURE 18 (CONTINUED)

```
                                                        |A3->                 |
GTGCTGAAGC AAGTCGTGAT AAAGCTTTTG CAGTATCAAA AGATTTAGCA GATAAATTGG    1140
  A  E  A   S  R  D   K  A  F  A   V  S  K   D  L  A   D  K  L  A    273

CAGCTAAAAC AGCAGAAGCT GAAAAGTTAA TGGAAAACGT TGGTAGTCTA GACCGCTTGG    1200
  A  K  T   A  E  A   E  K  L  M   E  N  V   G  S  L   D  R  L  V    293

TAGAGTCTGC AAAACGTGAA ATGGCTCAAA AATTAGCAGA AATTGATCAA TTAACTGCTG    1260
  E  S  A   K  R  E   M  A  Q  K   L  A  E   I  D  Q   L  T  A  D    313

ATAAGGCTAA GGCTGATGCA GAGCTTGCAG CTGCAAATGA CACCATTGCA TCACTTCAAA    1320
  K  A  K   A  D  A   E  L  A  A   A  N  D   T  I  A   S  L  Q  T    333

CAGAGCTAGA AAAAGCTAAG ACAGAGCTTG CTGTTTCAGA GCGTTTGATT GAATCAGGCA    1380
  E  L  E   K  A  K   T  E  L  A   V  S  E   R  L  I   E  S  G  K    353

          B1->                                  |
AACGTGAAAT TGCTGAGCTA CAAAAACAAA AAGATGCTTC TGATAAGGCT TTAGTAGAAT    1440
  R  E  I   A  E  L   Q  K  Q  K   D  A  S   D  K  A   L  V  E  S    373

             B2->                               |
CACAAGCTAA TGTAGCAGAG CTTGAAAAAC AAAAAGCAGC ATCAGATGCT AAGGTAGCAG    1500
  Q  A  N   V  A  E   L  E  K  Q   K  A  A   S  D  A   K  V  A  E    393

AGCTTGAAAA AGAAGTTGAA GCTGCTAAAG CTGAGGTTGC AGATCTTAAA GTACAATTAG    1560
  L  E  K   E  V  E   A  A  K  A   E  V  A   D  L  K   V  Q  L  A    413

CTAAGAAAGA AGAAGAGCTT GAAGCCGTTA AGAAGGAAAA AGAAGCGCTT GAAGCTAAGA    1620
  K  K  E   E  E  L   E  A  V  K   K  E  K   E  A  L   E  A  K  I    433

TTGAAGAGCT CAAAAAAGCT CATGCTGAGG AACTTTCAAA ACTTAAAGAA ATGCTTGAGA    1680
  E  E  L   K  K  A   H  A  E  E   L  S  K   L  K  E   M  L  E  K    453

AGAAAGACCA TGCAAATGCA GATCTTCAAG CAGAAATCAA TCGCTTGAAG CAAGAGCTAG    1740
  K  D  H   A  N  A   D  L  Q  A   E  I  N   R  L  K   Q  E  L  A    473

CTGACAGGAT TAAGTCATTG TCACAAGGTG GTCGTGCTTC ACAAACAAAC CCAGGCACTA    1800
  D  R  I   K  S  L   S  Q  G  G   R  A  S   Q  T  N   P  G  T  T    493

                                                    M->
CAACTGCTAA AGCAGGTCAA TTGCCATCTA CTGGTGAGTC TGCTAACCCA TTCTTCACTA    1860
  T  A  K   A  G  Q   L  P  S  T   G  E  S   A  N  P   F  F  T  I    513

                                                    |C->
TTGCAGCTCT TACTGTCATC GCTGGTGCTG GTATGGCTGT GGTGTCTCCT AAACGCAAAG    1920
  A  A  L   T  V  I   A  G  A  G   M  A  V   V  S  P   K  R  K  E    533

AAAACTAAGC TATTTCCTCT TTCCCCAATG GACAATAGCC GAAATAATAG AGCGACTATC    1980
  N  *                                                              534

GTTCTAACAC AAAAGCAACA GTCTCCTGTC TGTTGCTTTT TGTGATATTA GGGCTCATCA    2040
           >>>>>>>>>>>>>>          <<<<<<<<<<<<<

GTCTAGGCTA ATGGTTTTCT GCGCTTTATC TGCAG                               2075
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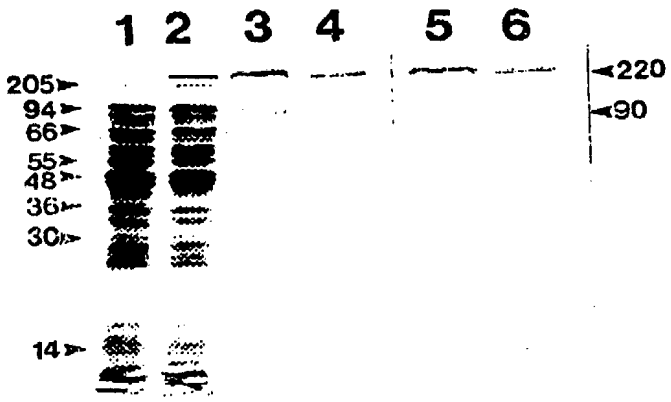

FIGURE 19

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IE 97/00046

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/31 C07K14/315 C12N1/21 A61K39/09 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GALAN J E ET AL: "MOLECULAR ANALYSIS OF THE M PROTEIN OF STREPTOCOCCUS EQUI AND CLONING AND EXPRESSION OF THE M PROTEIN GENE IN ESCHERICHIA COLI" INFECTION AND IMMUNITY, vol. 55, no. 12, December 1987, pages 3181-3187, XP000645178 cited in the application see abstract see page 3183, left-hand column, paragraph 3 - page 3186, right-hand column, paragraph 2 --- -/--	1-14

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 October 1997	21.10.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Montero Lopez, B

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intern nal Application No
PCT/IE 97/00046

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SUSANNE R. TALAY ET AL.: "Structure of a group C streptococcal protein that binds to fibrinogen, albumin and immunoglobulin G via overlapping modules" BIOCHEMICAL JOURNAL, vol. 315, no. 2, 15 April 1996, pages 577-582, XP002040105 see abstract see page 578, left-hand column, paragraph 1 - right-hand column, paragraph 1 see page 578, right-hand column, paragraph 5 - page 579, right-hand column, paragraph 2 see page 581, right-hand column, paragraph 3 - page 582, left-hand column, paragraph 1 -----	1-14